Male Subfertility and Genetics

From clinic to gene and back

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de Medische Wetenschappen

Proefschrift

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Levend in de tijd van het existentialisme, die men het 'tijdperk van het wantrouwen' noemde, vatte Sartre het menselijk bestaan samen onder een paradoxale formule: de mens is een wezen, veroordeeld tot de vrijheid.

Dat klopte in een tijd waarin eenzaamheid en engagement de gevleugelde woorden waren. Onze tijd echter wordt beheerst door de parolen coöperatie en communicatie. Daardoor zitten we gevangen in een andere paradox:

Wij zijn veroordeeld tot het vertrouwen. Dat betekent niet dat we als blinden de toekomst van de monsterlijke technologie tegemoet rennen, maar met een onbegrensde vrijheid en op basis van huidige kennis discussiëren over de risico's van ontwikkelingen die allang zijn begonnen.

Uit: Peter Sloterdijk, *Le Monde*, 9 oktober 1999

Contents

List of Abbreviations

AR	Androgen receptor
ART	Artificial reproductive techniques
AZF	Azoospermia factor
CBAVD	Congenital bilateral absence of the vas deferens
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane regulator
CUAVD	Congenital unilateral absence of the vas deferens
DAZ	Deleted in azoospermia
DAZLA	Deleted in azoospermia like autosome
ET	Embryo transfer
FSH	Follicle stimulating hormone
ICSI	Intracytoplasmic sperm injection
IUI	Intrauterine fertilisation
IVF	In vitro fertilisation
LH	Luteinizing hormone
MESA	Microsurgical epididymal sperm aspiration
MtDNA	Mitochondrial DNA
NRY	Non-recombining region of the Y chromosome
OAT	Oligoasthenoteratozoospermia
PCR	Polymerase chain reaction
PESA	Percutaneous sperm aspiration
SSCP	Single strand confirmation polymorphism
STS	Sequenced tagged sites
TESE	Testicular sperm extraction
WHO	World Health Organization

General Introduction

General Introduction

Fertility has been an important issue in all recorded history of mankind. Ample proof of this may be seen in the worshipping of diverse fertility gods in different cultures. For example, the ancient Egyptians already made offerings to fertility gods, like Hathor and created phallus symbols. From a biological and population point of view reproduction is important in species as a compensation for death.

To remain without offspring is a grave burden for couples facing fertility problems. In 50 percent of fertility problems a male factor is involved. Even now the aetiology of male subfertility remains unclear in about 40 percent of cases. This is due to the fact that male subfertility studies were not undertaken systematically before the 1960s.

Since the sexual revolution it gradually became easier to perform studies on male fertility. This factor, and the evidence-based medicine as a pervasive force in all fields of medicine, has stimulated the research on male subfertility during the last decades.

There are two main causes for the current interest in the genetics of male factor subfertility. Firstly we are witnessing an explosion of knowledge on the genetics of reproductive medicine. Secondly, since the introduction of intracytoplasmic sperm injection (ICSI) there has been concern about its risks to the offspring especially genetic abnormalities. These aspects have stimulated us to study the clinical and molecular genetic aspects of male subfertility and its clinical implications.

Male Subfertility

Prevalence and aetiology

In the Netherlands, every one in seven couples does not achieve a pregnancy within one year of active sexual intercourse. At the end of their reproductive lifespan, 2 to 7 percent remain childless. Subfertility may have various causes in either partner. According to the World Health Organization (WHO, 1992), in 20 percent of all cases the problem is predominantly male and in 38 percent predominantly female.

Male fertility requires normal spermatogenesis, epididymal maturation, storage of sperm, accessory gland function and sperm transport together with appropriately timed intercourse. The suboptimal reproductive capacities of one partner may become evident in

case of subfertility of the other partner. Male subfertility is frequently associated with a gross reduction in the number of sperm (oligozoospermia) or less frequently with the complete absence of sperm in the ejaculate (azoospermia), with abnormal motility of sperm (asthenozoospermia) or with abnormal morphology of the sperm (teratozoospermia). The results of semen analysis are an important indication of (sub-) fertility in males but are usually not considered to be sufficient for the diagnosis. The WHO defined male subfertility as lack of conception after at least 12 months of unprotected intercourse in combination with at least two separate semen samples, collected several weeks apart, that are not meeting the WHO criteria. However, many investigators (van Dop *et al.,* 1998; Cohlen, 1997) have suggested less strict criteria than the WHO.

Table I. Reference range of semen parameters (WHO, 1992)

Semen Parameter	**Reference range**
Seminal plasma volume	> 2.0 ml
Concentration	> 20.0 x 10^6 per ml
Total progressive motility	> 50%
Normal morphology	> 10%

Male subfertility may be categorised as: pretesticular, testicular and post testicular factors (see for review; de Kretser, 1997). Infection, trauma, cryptorchidism, varicocele, chemotherapy, malignancy, vasectomy and genetic factors are important causes of male factor subfertility. However, in up to 40% of subfertile males the aetiology remains unknown. In these cases the question arises whether this could be explained by (yet unknown) genetic factors.

Andrological investigation traditionally relies on a thorough history, physical examination, endocrinologic work-up and semen analysis. Since the introduction of ICSI this andrological investigation has been widened with the search for genetic causes of male subfertility. A genetic origin of male subfertility may explain the aetiology and may also indicate possible risks to the ICSI offspring before treatment.

Genetic aspects of male subfertility

Male factor subfertility can occur either as an isolated disorder or within the framework of a known complex disorder or syndrome. Male subfertility can be caused by diverse genetic abnormalities. In the following sections (1) the familial occurrence of male subfertility (2) chromosomal abnormalities in male subfertility (3) microdeletions of the Y-chromosome and (4) monogenetic factors and candidate genes in male subfertility will be discussed.

The familial occurrence of male subfertility

In 1984 Budde *et al.,* performed the first family study on male subfertility. An increased frequency of fertility problems among brothers of subfertile men was described. They hypothesised that this could be caused by an autosomal recessive or X-linked inheritance. Lilford *et al.,* 1994 also showed an increased frequency of male fertility problems among brothers. In this study segregation analysis showed that male subfertility has an autosomal recessive mode of inheritance in over half of the cases and that probably several genes are involved. Recently, a study by Meschede *et al.,* (2000) confirmed the familial clustering of male subfertility, but they proposed a multifactorial inheritance. In all these studies the prevalence of male fertility problems among families in the population is unknown. Therefore we started a survey on the familial occurrence of male subfertility. In this study (**Chapter 1**) we will also investigate the accuracy of the family history on fertility problems. To study the supposed pattern of inheritance in male subfertility the prevalence of subfertility among relatives of subfertile men will be compared with this prevalence among relatives of the randomly selected men (**Chapter 2**). We will also demonstrate possible phenotypic differences between subfertile men with and without a positive family history. With the help of the clinically well-defined group of severe subfertile men we identified a family showing 5 subfertile maternal nephews. In **Chapter 3** we report on this family and describe the results including the X-linkage analysis in this family. The group of well-defined severe subfertile men described in chapter 2 is the clinical basis for molecular genetic studies, presented in **Chapter 4** and **Chapter 5**.

Chromosomal abnormalities in male subfertility

Starting with the description of Klinefelter syndrome by Jacobs and colleagues (1959) many studies have examined the role of chromosomal abnormalities in male subfertility. Reported frequencies of chromosomal aberrations range from 2.2% in subfertile men (sperm count < $20x10^6$ per ml) and 6.0% in males with severe oligozoospermia to 19.6% in azoospermic men (Chandley *et al.,* 1979). In a study by Tuerlings *et al.,* (1998) it was shown that 4.0% of the 1792 male ICSI candidates revealed a chromosomal abnormality, predominately sex chromosome aberrations (47,XXY) and Robertsonian translocations (between chromosomes 13 and 14). In general, the frequency of karyotypic abnormalities among subfertile males increases when the number of spermatozoa decreases.

Microdeletions of the Y chromosome

Tiepolo and Zuffardi (1976) were the first to describe the azoospermia factor (AZF) region of the Y chromosome based on the observation of cytogenetic deletions in infertile men. Many studies have investigated the role of this region in spermatogenesis. Until now it has been suggested that some of the genes in the Yq11 region control spermatogenesis (Ma *et al.,* 1993; Reijo *et al.,* 1995; Lahn and Page, 1997). This region can be divided in three nonoverlapping regions of the Y chromosome: AZFa, b and c (Vogt *et al.,* 1996).

Microdeletions of the Y chromosome occur in 1% to 29% of subfertile men (Van der Ven *et al.,* 1996; Foresta *et al.,* 1997; Kremer *et al.,* 1997). The frequency is dependent on the definition of male subfertility and on the choice of Sequence Tagged Sites used for screening. The AZFc locus containing the DAZ gene cluster is the most frequently deleted region of the Y chromosome in men with non-obstructive infertility (Kostiner *et al.,* 1998; Simoni *et al.,* 1998). Histologically these deletions are associated with various spermatogenetic alterations, such as: Sertoli Cell Only syndrome, maturation arrest and hypospermatogenesis.

After the introduction of ICSI, men with a microdeletion of the Y chromosome could father children despite their severe oligozoospermia or azoospermia, using ejaculated or surgical retrieved spermatozoa. Microdeletions in subfertile fathers as well as their sons, born after ICSI, have been reported (Kent-First *et al.,* 1996). In a clinically well characterised cohort of male ICSI candidates (Kremer *et al.,* 1997) showed that all 7 of the 111 men with a microdeletion in the AZFc region of the Y chromosome had severe oligozoospermia, but normal clinical andrological findings and normal FSH levels.

The candidate gene for male subfertility *DAZLA* (*D*eleted in *AZ*oospermia *L*ike *A*utosomal) on human chromosome 3 shares a high degree of homology with the DAZ gene (*D*eleted in *AZ*oospermia) on the Y-chromosome. It is suggested that the DAZ genes arose from the transposition of *DAZLA* to the Y-chromosome followed by repeated amplification and pruning (Saxena *et al.,* 1996). Its testis specific expression and its homology to DAZ support the role of *DAZLA* in spermatogenesis. Disruption of the *DAZLA* gene in mice leads to loss of germ cells in both ovary and testis and absence of gamete production, demonstrating that *DAZLA* is essential for the differentiation of germ cells in mice (Ruggiu *et al.,* 1997).

We hypothesised that mutations in the human *DAZLA* gene are responsible for some of the (familial) cases of male subfertility. In **Chapter 4** we describe the search for mutations in the *DAZLA* gene in severe subfertile men using Single Strand Confirmation Polymorphism (SSCP) and sequencing techniques.

Monogenetic factors and candidate genes in male subfertility

From animal studies in drosophila and mice, it is known that perhaps thousands of genes are involved in spermatogenesis (Hackstein et al., 2000). On the other hand, a number of well-known monogenic disorders present with male subfertility (see for review Tuerlings *et al*. 1998; Meschede, 1997; for reproductive endocrine review see Fauser, 1995). Monogenic disorders can cause male subfertility, but many of them are accompanied by other clinical symptoms such as an impaired general health or mental and sexual developmental delay.

One of the more common monogenic disorders involved in male factor subfertility is cystic fibrosis (CF) which is caused by mutations in the CFTR gene. Cystic Fibrosis (CF) is the most common autosomal disease in the western world. The classical CF symptoms are progressive lung disease and pancreatic insufficiency, elevated sweat electrolyte values and congenital bilateral absence of the vas deferens (CBAVD) resulting in obstructive azoospermia. Ninety seven percent of male patients with classical CF are infertile (azoospermia) due to bilateral absence of the vas deferens. It is now clear that specific mutations of the CFTR gene may manifest as isolated CBAVD in the absence of other symptoms. Approximately 80 percent of men with CBAVD without clinical symptoms of CF, have detectable mutations in the CFTR gene locus (Dumur *et al.,* 1996).

To date, little is known about the frequency and causes of isolated congenital unilateral absence of the vas deferens (CUAVD) or of cystic changes in the Wolffian duct system. In addition, we know little about the frequency of CFTR mutations in these men. The CFTR gene might play a direct role in spermatogenesis or sperm maturation (Casals *et al.,* 1995; Van der Ven *et al.,* 1996). They found that 14 out of 80 (17.5%) males with fertility problems due to variable abnormalities of sperm quality had at least one CFTR mutation. This was significantly higher than the expected CFTR carrier frequency, estimated at 4 percent, in their general population. These results could not be confirmed in a study by Tuerlings *et al.,* (1998a) on the frequency of CFTR mutations in male ICSI candidates with severe oligoasthenoteratozoospermia.

Some other candidate genes for male subfertility have been proposed like the Follicle Stimulating Hormone (FSH) receptor gene. FSH is considered to be essential for spermatogenesis, while Luteinizing Hormone regulates steroidogenesis. An inactivating point mutation of the FSH receptor gene has been reported in some men with elevated serum FSH concentrations and abnormal sperm parameters (Tapanainen *et al.,* 1997). In a study of 28 men with high FSH levels, no mutations were found in the FSH receptor gene (Tuerlings et al., 1998b).

Another candidate gene, the Y chromosomal gene USP9Y, has been studied recently. A de novo point mutation in this Y chromosomal gene USP9Y is described in an azoospermic man (Sun *et al.,* 1999).

Furthermore, Aiman *et al.,* (1979) postulated that defects in the *AR* gene could lead to partial or complete spermatogenic failure. This *AR* gene, located on the long arm of the X chromosome at Xq11-12, contains a polymorphic CAG repeat sequence in the first exon, which encodes a series of glutamine residues in the region of the AR protein associated with DNA (Brown *et al.,* 1989). In vitro, the length of the CAG repeat sequence is inversely correlated with *AR* transcriptional activity (Kazemi-Esfarjani *et al.,* 1995). A relatively short CAG repeat is linked to an increased risk of prostate cancer, an androgen-dependent tumour (Giovannucci *et al.,* 1997), whereas a length of 38-62 CAG repeats leads to Kennedy's disease, a condition associated with bulbar muscular atrophy, low virilization and depressed spermatogenesis (Nagashima, 1988; La Spada *et al.,* 1991). Several groups reported conflicting results on the role of the CAG repeat length in the *AR* gene in cases of male subfertility (Dowsing *et al.,* 1999; Dadze *et al.,* 2000). Because of these conflicting results we will report in **Chapter 5** on the hypothetical association between the

expansion of the CAG repeat of the *AR* gene and subfertility among a group of Dutch subfertile men.

Treatment of male subfertility

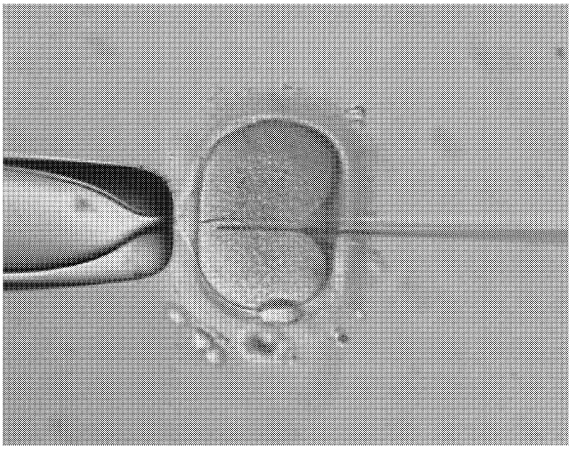

Figure 1. Injection of a single sperm into the oocyte: IntraCytoplasmic Sperm Injection

Prior to the era of assisted reproduction, most couples suffering from male subfertility had a relatively poor prognosis and chances of achieving a pregnancy were low to zero. Treatment of male subfertility was rarely evidence based and varied from variation in periods of abstinence, antibiotics in case of the presence of leukocytes in sperm, hormone therapy, functional foods, to surgical intervention in case of varicoceles. Many of these therapies are now regarded as questionable or obsolete (Nieschlag and Behre, 2000).
Only men with a deficiency of gonadotropic hormones may be successfully treated with hormones but this condition is a very rare cause of male subfertility. Low or absent production of gonadotropic hormones causes inadequate production of testosterone and leads to impotence and impaired spermatogenesis in the Sertoli cells. Primary deficiency of these gonadotropic hormones delays puberty; in these cases puberty can be induced by testosterone treatment. However, this treatment will not induce fertility. If offspring is desired they need to be treated with pulsatile Gonadotropin Releasing Hormones (GnRH) (Nieschlag and Behre, 2000). Another rare cause of hypogonadotropic hypogonadism is hyperprolactinemia, which can be treated with dopaminergic drugs. The treatment of couples facing male subfertility nowadays does not focus on improving sperm parameters,

but tries to get the sperm as close as possible to the oocyte. The lack of treatment options to improve semen parameters has stimulated the use of artificial reproductive techniques (ART) in male subfertility.

The ART techniques can be subdivided into two main groups; 1) in vivo techniques: Intra Uterine Insemination (IUI) and 2) in vitro techniques: In Vitro Fertilisation (IVF) and IntraCytoplasmic Sperm Injection (ICSI). Especially the introduction of ICSI has brought about a revolutionary change. Alternative options are adoption, donor insemination or accepting infertility. The couple and the physician have to weight out benefits and disadvantages of these treatments such as success rates, complications and cost-effectiveness.

The various treatment options and their success rates depend on diverse factors: female age, primary or secondary infertility and semen parameters. The amount of motile spermatozoa in the ejaculate and/or after preparation of the spermatozoa determines the treatment that is advised to the subfertile couple. In subfertile men with sperm concentrations below $1x10^6$/ml after preparation, the prognosis of IUI is very poor (Nieschlag and Behre, 2000). In a recent study by van Voorhis *et al.,* (2001) it is suggested that an average total motile sperm count of 10 million may be a useful treshold value to decide that a couple should be treated with IUI or IVF. One should consider that IUI is performed easily, requires minimal equipment and remains practically without complications. In comparison with more invasive and more costly treatments like IVF, the single treatment cycle with IUI is worse (Goverde *et al.,* 2000). In couples suffering from a slight form of male subfertility the efficacy of six consecutive with IUI is comparable to the efficacy of a single treatment of IVF, however with lower financial costs. Therefore, it is important to explain to couples that IUI must not be considered as one single treatment but rather as a series of successive trials.

In vitro fertilisation and embryo transfer

Louise Brown was the first child born after a successful in vitro fertilisation (IVF) and embryo transfer (ET) procedure (Steptoe and Edwards, 1978). The technique of IVF requires the collection of oocytes, initially by laparoscopy and later by transvaginal ultrasound-guided puncture of preovulatory follicles. Oocytes are obtained from the follicular fluid and incubated with motile sperm. Following fertilisation embryos are cultured and 2 or 3 days later they are transferred into the uterus (embryo transfer).

This treatment made it possible to treat couples with long-standing subfertility. In the early days IVF was indicated solely in case of tubal disease, later on endometriosis, unexplained subfertility and moderate male factor subfertility were induced. In a cochrane review study (van Rumste *et al.,* 2001) it is concluded that fertilisation rates are significantly better with ICSI than IVF in couples with borderline semen. Whereas the decision to perform ICSI rather than IVF is obvious in many situations, the choice may become difficult in borderline cases. The predictive accuracy of conventional semen analysis for sufficient fertilisation after IVF is not exactly known.

Intracytoplasmic sperm injection (ICSI)

Several procedures have been developed to overcome the fertilisation failure in severe oligoasthenozoospermia. These include partial zona drilling (Gordon and Talansky, 1986; Cohen *et al.,* 1989; Cohen *et al.,* 1991) and subzonal insemination (SUZI; Laws-King A. *et al.,* 1987). All these techniques were not very successful.

In 1992 the first report of ICSI resulting in a human pregnancy appeared (Palermo *et al.,* 1992). In the ICSI procedure the oocyte is fixed with the polar body visible at the top or bottom of a holding pipette. The technique has proven to be very successful in treating couples with severe male subfertility. As early as 1976, Uehara and Yanagimachi have shown microinjection of a single sperm into the cytoplasm of a retrieved oocyte. Worldwide thousands of children have been born after ICSI treatment and ongoing pregnancy rates of up to 35% per ICSI cycle have been reported (Van Steirteghem *et al.,* 1993). The indications for ICSI are severe oligoasthenoteratozoospermia (OAT, in the Netherlands less than $1x10^6$ motile sperm, WHO, 1992) or total fertilisation failure in two IVF procedures.

One of the main advantages of ICSI relates to the small number of sperm that is required (i.e. one per egg). In addition, ICSI can also be successful in case of microscopically abnormal sperm, total absence of motility or even in case of immature sperm (Nieschlag, 2001).

Surgical retrieval sperm

Originally, sperm was only retrieved from ejaculated semen or frozen-thawed semen. Soon after the introduction of ICSI it became evident that fertilisation can be achieved using epididymal sperm or even testicular retrieved sperm (Nagy *et al.,* 1995). In contrast using epididymal sperm in IVF had very poor fertilisation rates. Later, it was shown that children were born after ICSI using round spermatids (Tesarik *et al.,* 1995). Most recently, a pregnancy was reported after ICSI using a secondary spermatocyte (Sofikitis *et al.,* 1998). From these results it can be deduced that spermatozoa do not have to go through the full length of the genital tract before to be able to fertilise, but that also immature germ cells can establish fertilisation.

Epididymal sperm is retrieved by surgical needle aspiration (microsurgical epididymal sperm aspiration, MESA; or Percutaneous epididymal sperm aspiration, PESA) and testicular sperm by testicular sperm extraction (TESE). MESA is used in cases of obstructive azoospermia, while TESE can be used in cases of non-obstructive azoospermia as well. The only indication of using spermatids for ICSI may be in case of azoospermia with failure of spermatogenesis. There is only limited experience with surgically retrieved sperm, especially in using immature germ cells, and the number of children born after these techniques is small. As a consequence, very little is known about the (potential) risks. This is the reason that in The Netherlands it is not permissible at present to use spermatozoa from testicular origin and the use of epididymal sperm is only allowed in an experimental study.

Counselling and outcome of intracytoplasmic sperm injection

Counselling of male subfertility and ICSI

Several authors emphasised the importance of "genetic" counselling in ICSI candidates (Tuerlings et al., 1997; Pauer *et al.,* 1997). Counselling the ICSI couple on the origin of male subfertility, the genetic aspects of male subfertility and the outcome of ICSI can help them to make their own and well-informed decision on their reproductive future. The information on the origin of male subfertility and the genetic aspects are discussed above. The couple should also be informed about the present knowledge on the outcome of ICSI. Potential problems related to ICSI may be divided into two categories (Patrizio, 1995).

1) Problems related to the ICSI technique: **(a)** the manipulative procedure itself, **(b)** injection of foreign material, such as plasmid DNA, infectious particles and paternal mitochondria, **(c)** mechanical activation of the oocyt and **(d)** exclusion of the pre-zygotic selection processes.
2) Problems not directly related to the ICSI technique (*the use of abnormal sperm*): **(a)** an increased frequency of constitutional chromosomal abnormalities (Tuerlings *et al.,* 1998c) and/or **(b)** chromosomal abnormalities in sperm (Moosani *et al.,* 1995), and **(c)** an underlying 'genetic' disorder which causes male subfertility (e.g. cystic fibrosis and Y chromosome deletions) (Dumur *et al.,* 1996; Kremer *et al.,* 1997), **(d)** gamete maturation could be impaired in epididymal or testicular sperm (Ariel *et al.,* 1994).

The conditions mentioned in the second group mainly concern the use of ICSI without understanding the exact aetiology of the fertility problem.

One of these theoretical risks has prompted us to investigate its nature. There is a risk that ICSI increases the transmission of paternal mitochondrial, bypassing the supposed mechanism (Kaneda *et al.,* 1995) by which paternal mtDNA is removed from the embryo. Knowledge of the fate and transmission of paternal mitochondrial DNA is important since mutations in mitochondrial DNA have been described in oligozoospermic males. Transmission of this mutated mitochondrial DNA may lead to mitochondrial DNA diseases to children born after this technique. In **chapter 6** we describe this study on the origin of mtDNA in placenta, umbilical cord, blood and buccal swabs of children born following ICSI. After the counselling procedure a couple has to make a decision on their reproductive future. If the genetic investigation revealed no abnormalities they have to make their

decision on the current knowledge on the safety of the ICSI procedure. But if there is a genetic aberration like a chromosomal abnormality in the subfertile man the couple also has to take into account the possible risks related to this chromosomal abnormality. For example, carriers of balanced translocations can produce offspring with either a normal, chromosomally balanced or a chromosomally unbalanced karyotype. In the latter case this can result in an early developmental embryonic arrest, a spontaneous abortion, a still birth or birth of a child with a combination of (multiple) congenital anomalies and psychomotor retardation in any degree. In a study by Giltay *et al.,* (1999) it is shown that despite the genetic risks related to a chromosome abnormality in subfertile men, the majority (56%) of the couples did not refrain from the ICSI treatment.

Couples dealing with microdeletions of the Y chromosome also have to make decisions on their reproductive future. Since the introduction of ICSI these men can father children and presumably transmit the deletion as well as the related fertility problem to their sons. These couples can choose for ICSI, artificial insemination with donor insemination or no treatment. In **chapter 7** we report on the decision-making and the relationship between some aspects of the process of genetic counselling and the final decision.

Outcome and Follow-up of ICSI

The introduction of ICSI in 1992 was embraced as a major breakthrough for the treatment of male factor subfertility. It should be noticed that randomised clinical trials and data collection on the ICSI offspring did not accompany the rapid introduction of ICSI. Up till now there is only a limited number of studies on the follow-up of ICSI. Most papers illustrate the success rate of ICSI treatment, but there is no information at all except some case reports about success rates in couples with severe oligozoospermia due to microdeletions of the Y chromosome. In **chapter 8** we describe the outcome of ICSI treatment using sperm from a men with a microdeletion of the Y chromosome compared with the ICSI treatment using sperm from oligozoospermic men without this deletion.

At this moment most follow-up studies show no increased risk of congenital abnormalities after ICSI. However Bonduelle *et al.,* (1998a) report that there is a significant increase of de novo chromosomal abnormalities (1.66%) after ICSI, mainly sex chromosomal abnormalities (0.8%). This increased frequency may be caused by (1) the increased frequency of aneuploidy in spermatozoa (Ohashi *et al.,* 2001) or (2) the non-random positioning in human sperm following ICSI (Terada *et al.,* 2000).

Reclassification of birth defects of the prospective follow-up study from Brussels showed an increased risk of having a major birth defect (Kurinczuk *et al.,* 1997). There are also controversial results from studies on the development of children born after ICSI. Bowen *et al.,* 1998 showed an increased risk of mild delays in mental development at 1 year in children, especially in boys, conceived by ICSI. These results are not confirmed in a larger study by the Brussels group (Bonduelle et al., 1998b).
More follow-up data on ICSI should be evaluated because of theoretical risks, the novelty of the procedure and the limited number of controversial follow-up results. Therefore we report on the safety of ICSI by retrospectively comparing data of ICSI to IVF in **chapter 9**.

Scope of the thesis

In this thesis we study the genetic aspects of male subfertility in the following three parts.

Part I: In this part we present the results of the family studies in male subfertility.

Part II: We continue in this part with our molecular genetic search for genes involved in male subfertility. An X-linkage analysis and the investigation of the DAZLA and the androgen receptor gene will be shown.

Part III: In this last part we will describe the clinical implications of these genetic factors in male subfertility like counselling, decision making and the outcome of ICSI.

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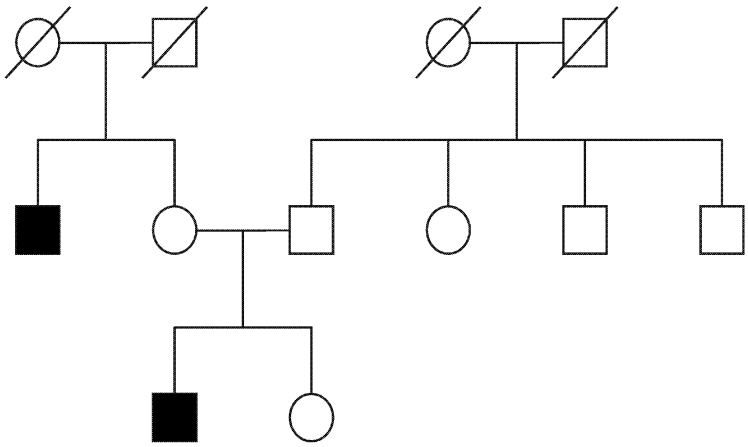
Part I

Family studies in male subfertility



Chapter 1

A survey on the prevalence of subfertility among relatives using a family history: Underestimation due to "Taboo Bias"

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Submitted

Abstract

Objective: To determine the prevalence of male subfertility among relatives in the population using a family history. Subsequently we studied the validity of the family history used in this survey.

Design: Survey

Setting: Questionnaires were sent to a group of 474 randomly selected men, aged 25-40 years, to collect data on subfertility among themselves and their relatives. A non-responder study and a personal interview were performed to evaluate the accuracy of the data obtained in this survey.

Main outcome Measure: Prevalence of subfertility

Results: Two hundred and forty three men (51.3%) completed the questionnaire. The reported prevalence of subfertility among the different relatives is significantly lower than among responders. Among brothers the reported prevalence is significantly five times lower, 3.6% versus 15.3% respectively. The non-responder study and the personal interview showed that these differences were not caused by a low response to this survey or by the use of a questionnaire instead of a personal interview.

Conclusions: Subfertility among relatives is underestimated using a family history, probably due to the taboo on discussing subfertility. Knowledge of subfertility travels selectively among families causing substantial misclassification. Clinicians and researchers should be aware that family history taking is likely to lead to underestimation of subfertility among relatives.

Introduction

In the Netherlands, every one in seven couples (14.2%) does not achieve a pregnancy within one year of active sexual intercourse (Beurskens *et al.,* 1995). Subfertility may have various causes in either partner. According to the World Health Organization (WHO, 1992), in 20 percent of all cases the problem is predominantly male and in 38 percent predominantly female. To date the causes of defective spermatogenesis often remains unknown. Recently, much attention has been given to genetic causes of male subfertility such as chromosomal aberrations (Tuerlings *et al.,* 1997), microdeletions of the Y-chromosome and other possible monogenetic causes. This attention is stimulated by the concern of potential transmission of genetic defects to the offspring via intracytoplasmic sperm injection (ICSI).

The family history can be used to study genetic aspects of male subfertility. In addition the family history can provide important clues for diagnosing, counseling and treatment of the patient. The prevalence of male subfertility among relatives when using a family history is unknown. Nevertheless, several authors suggest that the family history should be used in research and fertility practice (Lilford *et al.,* 1994; Meschede *et al.,* 2000; Tuerlings *et al.,* 1998). Therefore a survey was carried out on the familial occurrence of male subfertility to obtain knowledge on the prevalence of subfertility among relatives.

Materials & Methods

Questionnaire

We sent 474 questionnaires to a random sample of men, aged 25-40 years, living in the town of Boxmeer. Boxmeer is a typical Dutch municipality of average size and socio-economic status, with its own industry, agriculture and commerce. The city is situated in the southeast of the Netherlands and our university hospital is the tertiary medical center for this region. Using a questionnaire we collected information on medical and family history up to second-degree family members and we focussed on *subfertility*. In the Netherlands this is defined as a lack of conception after at least 12 months of unprotected intercourse.

The responders and their relatives were considered *proven fertile* if they had established a pregnancy or had children without reporting subfertility. People were considered *unproven fertile* if they did not report subfertility and had no children because (a) the childlessness was reported to be voluntary, (b) there was no heterosexual relationship, (c) the relative died before 25 years of age and (d) the relative was mentally retarded. Finally, when classification was still not possible, fertility was scored as *unknown*.
The prevalence of subfertility was calculated in the total group and in the subgroup at risk for subfertility. In this at risk subgroup (people who try or have tried to get pregnant) we excluded people with unproven fertility and unknown factors.

Non-responder study and side study

A reminder was sent to non-responders two weeks after the questionnaire. In addition we tried to phone all non-responding men. In this telephone inquiry the men were asked again to respond to the questionnaire. If they did not want to respond, non-responder data were gathered on (a) the reason for not responding, (b) their own fertility status and (c) if they had any relatives with subfertility.
A side study was performed to investigate the reproducibility of the data obtained with the questionnaire compared to data collected during a personal interview. We contacted 80 responders and asked them to participate in this personal interview.
Data from this non-responder investigation and the side study were used to investigate the accuracy of the data obtained in this survey.

Statistical methods

The prevalence of subfertility of different types of relatives was calculated. The prevalence was compared between responders and their relatives by calculation of odds ratios (OR) and their 95% confidence intervals (CI). In the side study we calculated kappa values, which indicate the chance-adjusted reproducibility of the data using a personal interview instead of a questionnaire. A kappa value of 1 indicates 100% reproducibility.

The study was performed simultaneously with a study on the prevalence of benign urological diseases and the Institutional Review Board approved these studies.

Table I. Fertility status among proband and relatives according to the family history

Family member	Proven fertility[a]	Fertility problems in total[b]	Fertility problems at risk subgroup[c]	Unproven fertility[d]	Unknown	Total	OR (95%CI)[e]
Proband	*116 (47.7%)*	***21 (8.6%)***	***21/137 (15.3%)***	*106 (43.6%)*	*0*	*243*	-
Brother	216 (56.7%)	8 (2.1%)	**8/224 (3.6%)**	142 (37.3%)	15 (3.9%)	381	0.2 (0.08-0.5)
Sister	237 (69.7%)	8 (2.4%)	**8/245 (3.3%)**	58 (17.1%)	37 (10.9%)	340	0.2 (0.07-0.5)
Maternal uncle	522 (82.5%)	16 (2.5%)	**16/538 (3.0%)**	86 (13.6%)	9 (1.4%)	633	0.2 (0.08-0.4)
Maternal aunt	550 (85.7%)	16 (2.5%)	**16/566 (2.8%)**	51 (7.9%)	25 (3.9%)	642	0.2 (0.08-0.3)
Paternal uncle	568 (85.0%)	8 (1.2%)	**8/576 (1.4%)**	70 (10.5%)	22 (3.3%)	668	0.08 (0.03-0.2)
Paternal aunt	555 (85.8%)	22 (3.4%)	**22/577 (3.8%)**	52 (8.0%)	18 (2.8%)	647	0.2 (0.1-0.4)
Non-Responder	*35 (34.7%)*	*3 (3.0%)*	***3/38 (7.9%)***	*58 (57.4%)*	*5 (5.0%)*	*101*	*0.8 (0.2-2.4)*

[a] Proven Fertility: People who are pregnant or have children without reporting fertility problems
[b] Fertility problems in total: People with or without children reporting fertility problems due to male and/or female or unknown factors with respect to the whole group
[c] Fertility problems in the at risk subgroup: People with or without children reporting fertility problems due to male and/or female or unknown factors with respect to the subgroup at risk for fertility problems (= fertile people and people with fertility problems)
[d] Unproven fertility: People with no heterosexual relationship or reporting that they are voluntary childless
[e] Odds rates (OR) and 95% confidence intervals (CI) of fertility problems among relatives versus fertility problems among probands

Results

In total 264 out of 474 (55.7%) men responded. Two-hundred and nine responders completed all questions, 34 men did not gave details on their second-degree relatives and 21 men only completed the medical history form and did not gave any details on the family history. In our study we included all 243 men, who completed the questionnaire on their first-degree relatives.

The mean age of responders was 33.3 (SD 4.4) years. An abnormal andrologic history was present in 16.1 % of the responders: a history of inguinal hernia in 19 (7.8%) men, male genital infection in 10 (4.1%), maldescended testis in 6 (2.5%), surgical correction of a varicocele in 3 (1.2%) and 1 (0.4%) man reported a torsion of his testicle.

Table I shows the fertility status of the responders and their relatives. Subfertility among responders was present in 8.6%. The prevalence of subfertility in the at risk subgroup was 15.3%.

The subfertility of the responders were reported to be due to: unknown factors (10/21; 47.6%), a female factor (4/21; 19.0%), a combined male and female factor (4/21; 19.0%) men and 3 men reported subfertility due to impaired semen quality (14.3%). No subfertility was reported among parents and grandparents.

The prevalence of subfertility among relatives are all significantly lower than among responders. Brothers show a 5 times lower prevalence of subfertility than responders. The unproven fertility was not significantly different among responders and their brothers, 43.6% and 37.3% respectively. The reasons for the unproven fertility among brothers were: (a) the brothers and their partners were voluntary childless (52.8%) and (b) if brothers had no partner (47.2%).

Non-responder study

One hundred and one men in the group of 210 non-responders (48.1%) participated in the telephonic non-responder study. The mean age of the non-responders was 32.8 (SD 4.8) years, which was not significantly different from the responders. Reasons for not responding were lack of time or a misconception that the study did not focus on people with unproven fertility.

Table I also shows the results from the non-responder investigation. The non-responders showed no significant difference in prevalence of subfertility versus responders, but had a

significantly higher prevalence (57.4%) of unproven fertility. The non-responders also reported a low prevalence of subfertility among *all* relatives (7.9%).

Side study

Forty of the 80 contacted responders participated in this study. Subfertility in responders and their relatives were reported more often using a personal interview. However, kappa values for the reported subfertility were high among responders, first-degree relatives and second-degree relatives: 0.96, 0.87 and 0.77 respectively. This indicates that a questionnaire shows a good reproducibility with the data obtained by a personal interview.

Discussion

In this study we report the prevalence of subfertility among relatives, when data are collected using the family history. The prevalence of subfertility among responders in this survey (15.3%) is in the same range as earlier reports (Beurskens *et al.,* 1995; Hull *et al.,* 1985). However, the reported prevalence of subfertility among relatives is significantly lower than among responders. For instance brothers, who do not differ significantly in age from the responders, show a 5 times lower prevalence of subfertility (OR 5.0; 2.0-12.5). The prevalence of subfertility is also significantly lower among second-degree relatives.

In our view the prevalence of subfertility among relatives in this study is invalid. As in the population we would expect at least the same prevalence of subfertility among relatives or higher, because of the possible genetic factor(s) involved in male subfertility. How can we then explain the lower reported prevalence of subfertility among relatives? The non-responder study and the personal interview showed that these differences were not caused by a low response to this survey or the use of a questionnaire.

The fertility of the young generation of the responders may be lower than the fertility of the older generation of second-degree relatives. This can be due to female subfertility caused by the higher female age when having the first child, nowadays. And in men it is suggested that during the last decades the semen quality is diminishing, which may cause an increase of male subfertility (Auger *et al.,* 1995; Carlsen et al., 1992 ; Irvine *et al.*,1996). But this hypothesis does not explain the difference found between responders and first-degree relatives.

Another explanation, for the differences found between responders and relatives, is misclassification. Relatives with subfertility may have been wrongly classified in this study as unproven fertile, proven fertile or unknown, because the responder is not aware of the presence of subfertility among relatives. This information bias or "taboo" bias can be caused by the difficulty of discussing subfertility with family members. For instance in research on donor insemination it is shown that the stigma associated with subfertility forces secrecy and lack of openness (Turner *et al.,* 2000).

Lilford *et al.,* (1994) and Meschede *et al.,* (2000) reported no subfertility among relatives in their fertile control groups and a higher frequency of subfertility among male relatives of infertile men. Underestimation of subfertility in the control group and a more accurate estimation of subfertility among relatives of subfertile men may cause this difference. The taboo bias involved in subfertility may also be the reason why permission to contact subfertile relatives in these family studies on male subfertility is often withheld.

The accuracy of the family history as a diagnostic tool has also been investigated in disorders like cancer, myocardial infarction and Alzheimer's disease. The general finding in these studies is that the family history can contribute to medical decision-making (Sijmons *et al.,* 2000; Dewey *et al.,* 2000; Kulig *et al.,* 2000). However, family history is not always reliable. Verification using medical records of relatives may be necessary to prevent misclassification and to correctly interpret the family history (Kee *et al.,* 1993).

In conclusion, news about subfertility may travel selectively throughout families causing information bias. In future research subfertility in families should be evaluated, if possible, by direct contact to family members. At this moment clinicians and researchers should be aware that taking a family history on subfertility is likely to underestimate subfertility among relatives.

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Chapter 2

Phenotypic characteristics of male subfertility and its familial occurrence

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Abstract

Genetic factors can cause male subfertility. A case-control study was carried out to investigate familial occurrence of male subfertility and the phenotypic characteristics of familial and non-familial male subfertility.

The medical data and family histories of 253 severe subfertile men, candidates for intracytoplasmic sperm injection, were compared to the data from 243 randomly selected men.

Genetic aberrations including a chromosomal abnormality or a microdeletion of the Y-chromosome are present in 13.8% of the severe subfertile men. The prevalence of male fertility problems among brothers and maternal uncles of subfertile men is significantly higher than among controls (brothers 10.4% versus 0.5% and maternal uncles 1.7% versus 0.2%). The phenotypes of subfertile men with a positive family history more often show normal levels of FSH and LH compared to the phenotypes of subfertile men with a negative family history. In addition, subfertile men with a positive family history have a lower percentage of motile sperm.

Male subfertility appears to have a familial occurrence, but misclassification hinders quantitative assessment of familial clustering. Furthermore the data suggest, that subfertile men with a familial occurrence of male subfertility more often have normal levels of FSH and LH.

Introduction

Male subfertility has a wide variety of causes. Some of these involve genetic abnormalities like: chromosome abnormalities (Tuerlings *et al.,* 1998), microdeletions of the Y chromosome (Tiepolo and Zuffardi, 1976; Reijo *et al.,* 1995; Kremer *et al.,* 1997) and diverse monogenetic factors such as Cystic Fibrosis Transmembrane conductance Regulator (CFTR) mutations in men with Congenital Bilateral Absence of the Vas Deferens (CBAVD) (Dodge, 1995). However, in the majority of subfertile men the aetiology remains unknown and the subfertility has to be classified as idiopathic (de Kretser, 1997; Dubin and Amelar, 1971). This idiopathic male subfertility may have a genetic origin considering the observed familial occurrence of male subfertility (Budde *et al.,* 1984; Lilford *et al.,* 1994; Meschede *et al.,* 2000).

From animal studies in drosophila and mice, it is known that perhaps thousands of genes are involved in spermatogenesis (Hackstein *et al.,* 2000). The family history may provide an important clue for these genetic causes of male subfertility and the patterns of inheritance. This knowledge may contribute to both genetic research and clinical management of male subfertility.

Therefore we studied (a) if subfertility occurs more often among relatives of subfertile men than among relatives of randomly selected men and (b) possible phenotypic differences between subfertile men with and without a positive family history.

Materials & Methods

Patients

We prospectively collected data from subfertile men visiting our fertility clinic from April 1998 to April 2000. We included 253 men with an azoospermia or a severe OligoAsthenoTeratozoospermia (OAT) with semen parameters that met our intracytoplasmic sperm injection (ICSI) criteria. Subfertile men are candidates for ICSI in our clinic if their ejaculate contains less than 1.0×10^6 spermatozoa with propulsive motility (WHO, 1992). Patients with a previous sterilization or a testicular malignancy in their medical history were excluded from this study.

At our outpatient clinic we collected the medical data, the family history and performed a physical examination. All cases of cryptorchidism or disturbance in descending of one or both testicles were scored as maldescended testes. We took blood samples for measurements of Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Testosterone, chromosome analyses and screening for microdeletions of the Azoospermia Factor (AZF) a, b and c region of the Y-chromosome (Hoefsloot *et al.,* 1997). In cases of obstructive azoospermia, the patient was directed to the department of Urology for further investigation. When a patient was diagnosed with a Congenital Unilateral/Bilateral Absence of the Vas Deferens (CU/BAVD) screening of CFTR mutations was performed.

Before the visit to our outpatient clinic, a questionnaire was sent to the subfertile men to collect information on their family history, specifically focussing on fertility problems. All patients provided us family history information on first and second-degree family members. During the visit we specifically asked the patient about involuntary childlessness and/or fertility problems in the family. Family members were considered *proven fertile* if they had established a pregnancy or had children without reporting a fertility problem. They were scored as *subfertile* when they reported fertility problems, with or without children, due to male and/or female or unknown factors. People were considered *unproven fertile* if they did not report fertility problems and had no children because (a) the childlessness was reported to be voluntary, (b) there was no heterosexual relationship, (c) the relative died before 25 years of age or (d) the relative was mentally retarded. Finally, when classification was still not possible, fertility was scored as *unknown*.

When the patient could not give the actual cause of childlessness of a family member we asked him to contact that family member in order to obtain the information. When the cause was a male factor we asked the patient to inform his relative about the study and to request his permission for a telephone call by one of the researchers. When the family member confirmed the male factor we asked him to undergo the same investigations as the patient in order to describe the phenotype.

Controls

We sent the same standardised questionnaire we used for the patients, to 474 randomly selected men, aged 25-40 years, living in the city of Boxmeer. In our study we included all men who completed the questionnaire giving details on their first-degree relatives, 243 of 474 or 51.3%. Details are described in the survey on the familial occurrence of male subfertility (van Golde *et al.,* 2001, unpublished data).

Statistics

For both groups we only included full siblings and relatives in the analyses. Differences between patients and controls were calculated with Odds ratios (OR) and their 95% confidence intervals (95 % CI). The statistical software package SAS version 6.12 (SAS, Cary NC) was used for analyses.

The study was approved by the local Institutional Review Board.

Results

Patients' clinical characteristics

The patient group consisted of 46 men with an azoospermia, 10 men with an asthenozoospermia and 197 men had an OAT. The most frequently encountered clinical abnormality was maldescended testes (n = 69), followed by varicocele (n=34), surgical correction of an inguinal hernia (n=32) and a history of male adnexitis (n = 26). The mean ages of the patients and the controls were similar: 33.0 years ± SD 4.5 years and 33.3 years ± SD 4.4 years.

Patients' genetic abnormalities

There were 35 patients with 36 genetic abnormalities among the 253 patients (13.8%), Table I. One man had two genetic defects: he suffered from Kartagener's Syndrome and he had a sex chromosomal abnormality, 46, X inv(Y) (p11.2;q11.23). The frequency of microdeletions of the Y-chromosome in this patient group was 0.8%.

Table I. Genetic aetiology of the fertility problem

Type of genetic abnormalities	Number of patients
Autosomal structural chromosome abnormality[a]	6
Sex chromosome abnormality[b]	6
Microdeletion of the Y-chromosome	2
Syndromal disorder with male subfertility[c]	3
CFTR[d] mutations in CBAVD[e]	14
CFTR[d] mutations in CUAVD[f]	2
Globozoospermia	3

[a] Robertsonian translocations [2], reciprocal translocation [3], inversion [1]

[b] 47,XXY [3]; 47,XY [1]; 45,X/46,XY [1]; 46,X,inv(Y)(p11.2;q11.23) [1]

[c] Kartagener's syndrome [2], Klippel-Feil Syndrome [1]

[d] CFTR = Cystic Fibrosis Transmembrane conductance Regulator

[e] CBAVD = Congenital Bilateral Absence of the Vas Deferens

[f] CUAVD = Congenital Unilateral Absence of the Vas Deferens

Family history

The prevalence of male fertility problems is significantly higher among brothers and maternal uncles of subfertile men than among brothers and maternal uncles of controls. The prevalence of fertility problems is 18/173 (10.4%) among brothers of subfertile men versus 1/222 (0.5%) among brothers of controls: OR= 26.0 (95% CI: 3.4-196.8). Among maternal uncles the prevalence of fertility problems is 9/523 (1.7%) and 1/534 (0.2%) respectively: OR=9.3 (95% CI: 1.2-73.9). There is also a statistically significant difference between the prevalence of male fertility problems among maternal and paternal uncles of subfertile men 9/253 (1.7%) versus 2/582 (0.3%): OR=5.0 (95% CI: 1.0-33.7).

No statistically significant differences were found between the other family members of patients and controls. Our study design did not focus on the fertility status of cousins. However 10 patients reported that they had one or more cousin with a male subfertility problem. These 16 cousins were 15 sons of maternal aunts and only 1 son of a paternal aunt. In the control group, no cousins were reported to have a fertility problem. The group of subfertile men have fewer siblings than the controls 2.4 and 3.0 respectively, mean difference: 0.6 (95%CI: 0.1-0.9). They have fewer brothers, mean difference: 0.3 (95%CI: 0.1-0.6).

Phenotypic characteristics of subfertile men

The main clinical characteristics of the 253 subfertile men, such as testis volume, semen analysis and hormone measurements are shown in Table II. The group is divided into men with and men without a positive family history. Family history is considered positive when one or more brother(s) or maternal uncle(s) reported male subfertility.

We found statistical significantly lower FSH- and LH-levels among men with a positive family history. The difference in FSH-levels was 2.5 IU/l (95%CI: 0.3-4.7) and in LH-level 1.6 IU/l (95%CI: 0.9-2.2). Subfertile men with a positive family history more often showed normal levels of FSH and LH. Moreover, subfertile men with a positive family history have a lower number of motile sperm, with a difference of 6.3% (95% CI: 0.9-11.6).

As shown in Table III, we were able to obtain the phenotype of 12 male relatives of 12 patients. In the other 15 patients with a positive family history we were not able to obtain direct information. Non co-operation is mostly present among maternal uncles of patients. The reasons for the patients not to contact their family members were: the patients did not want to inform his family members about their own fertility problem or they did not want to confront their relatives with questions concerning fertility.

For some of the genetic aberrations we found, a brother or uncle of the concerning patient had the same aberration (see families 1 and 2). Two patients and their maternal uncles had high FSH-levels. Apart from the cases of globozoospermia and CBAVD no specific phenotype could be recognised.

Genetic aetiology of male subfertility

A genetic origin of male subfertility can be considered in subfertile men when there is: (a) a genetic abnormality as defined in Table I (13.8%) or (b) a positive family history (12.1%). Hypothetically, a genetic origin can be considered in about 1 out of 4 subfertile men.

Table IIa. Testis volume, semen analyses and hormone measurements of the cases with a **positive** family history

	Testis Volume (ml) Right	Testis Volume (ml) Left	Sperm Concentration (10^6/ml)	Motility (% propulsive)	FSH (IU/l)	LH (IU/l)	Testosterone (nmol/l)
Reference range	*>15*	*>15*	*≥ 20*	*≥ 50*	*2.0-7.5*	*1.8-9.5*	*11-45*
Azoospermia (n = 7)	16.43 ± 6.27 (15.0)	16.14 ± 5.24 (15.0)	0	0	6.90 ± 4.08* (5.4)	3.43 ± 1.05* (3.5)	16.23 ± 5.81 (14.0)
Asthenozoospermia (n = 2)	20.00 ± 7.07 (20.0)	20.00 ± 7.07 (20.0)	32.50 ± 17.67 (32.5)	3.00 ± 2.83* (3.0)	8.70 ± 2.26 (8.7)	4.85 ± 0.78 (4.9)	13.00 ± 0.00 (13.0)
OAT (n = 17)	18.71 ± 5.37 (18.0)	17.12 ± 4.85 (15.0)	3.82 ± 3.96 (2.4)	16.56 ± 9.26 (15.0)	7.16 ± 5.51 (5.4)	3.43 ± 0.96* (3.3)	17.33 ± 6.88 (16.5)
All (n = 26)	16.19 ± 5.59 (18.0)	17.08 ± 4.96 (15.0)	5.00 ± 9.54 (1.6)	15.06 ± 9.76* (15.0)	7.21 ± 4.88* (5.5)	3.54 ± 1.01* (3.5)	16.67 ± 6.29 (16.0)

Table IIb. Testis volume, semen analyses and hormone measurements of the cases with a **negative** family history

	Testis Volume (ml) Right	Testis Volume (ml) Left	Sperm Concentration (10^6/ml)	Motility (% propulsive)	FSH (IU/l)	LH (IU/l)	Testosterone (nmol/l)
Reference range	*>15*	*>15*	*≥ 20*	*≥ 50*	*2.0-7.5*	*1.8-9.5*	*11-45*
Azoospermia (n = 39)	15.29 ± 6.39 (15.0)	14.55 ± 6.45 (15.0)	0	0	14.48 ± 13.17* (9.7)	6.68 ± 6.14* (4.8)	17.16 ± 8.10 (17.0)
Asthenozoospermia (n =8)	18.75 ± 3.54 (20.0)	18.13 ± 3.72 (17.5)	51.88 ± 34.12 (37.5)	14.50± 7.96* (15.0)	5.75 ± 3.73 (3.95)	3.70 ± 1.67 (3.25)	14.94 ± 5.13 (14.0)
OAT (n = 180)	16.22 ± 4.08 (15.0)	15.55 ± 4.29 (15.0)	2.74 ± 3.31 (1.5)	21.66 ± 16.79 (20.0)	8.91 ± 5.52 (7.1)	4.86 ± 2.41* (4.2)	16.41 ± 6.79 (15.0)
All (n = 227)	16.15 ± 4.55 (15.0)	15.47 ± 4.73 (15.0)	4.00 ± 11.40 (1.0)	21.35 ± 16.56* (20.0)	9.68 ± 7.53* (7.1)	5.09 ± 3.31* (4.2)	16.46 ± 6.93 (15.0)

Values are presented as Mean ± SD; values between parentheses are medians

* Statistically significant differences between subfertile men with or without a positive family history

Table III. Phenotypes of subfertile males in positive case families

ID	Member	Testes volume (R/L)	Spermatozoa ($*10^6$)	Motility (%)	FSH (IU/l)	Karyotype	Clinical remarks
Reference Range		*>15 ml*	*≥ 20*	*≥ 50*	*2.0-7.5*		
1p	Proband	12/12	2.8	15%	2.8	46, XY	Globozoospermia
1b	Brother	15/15	4.0	10%	8.5	46, XY	Globozoospermia
2p	Proband	25/25	azoospermia	-	2.5	46, XY	CBAVD
2b	Brother		azoospermia	-	NA	46, XY	CBAVD
3p	Proband	15/15	1.3	20%	4.7	46, XY	Inguinal hernia, epididymitis, maldescended testis
3b	Brother	12/10	0.1	-	11.0	46, XY	Maldescended testis
4p	Proband	15/15	4	30%	7.9	46, XY	
4b	Bother	NA	azoospermia	-	Normal	NA	Secondary subfertility, obstruction, CUAVD
5p	Proband	25/25	7.5	5%	7.1	46, XY	asthenozoospermia
5b	Brother	20/20	30	1%	8.9	46, XY	
6p	Proband	15/15	4	30%	8.8	46, XY	Hydrocele
6b	Brother	15/12	NA	NA	20.8	46, XY	Maldescended testis
7p	Proband	15/15	45	<1%	10.3	46, XY	Varicocele
7b	Brother	15/15	OAT/ azoospemia	-	2.3	46, XY	Persistent OAT after cystectomy of prostate cyste
8p	Proband	30/25	6	30%	4.4	46,XY,inv(7) (q22.1q31.3)	Varicocele, inversion of chromosome 7 in more family members
8b	Brother		Asthenospermia	NA	NA	NA	
9p	Proband	15/15	Azoospermia	-	3.8	46,XY	CBAVD
9b	Brother		Azoospermia	-	NA		
10p	Proband	15/15	Azoospermia	-	13.0	46, XY	hypogonadism hypergonadotrope
10mu	Maternal Uncle	15/15	Azoospermia	-	27.9	46, XY	Maldescended testis
11p	Proband	12/12	10	15	4.5	46, XY	Globozoospermia, Maldescended testis
11mu	Maternal Uncle	15/20	45	20	4.2	46, XY	Globozoospermia
12p	Proband	15/15	Azoospermia	-	24.7	46, XY	Maldescended testis
12mu	Maternal Uncle	12/12	0.4	30%	14.9		

NA = not available
CU/BAVD = congenital unilateral/bilateral absence of the vas deferens
p = proband, b = brother, mu = maternal uncle

Discussion

Male subfertility may have a familial occurrence. The higher prevalence of male subfertility among brothers and maternal uncles of subfertile men suggest this. The increase of male subfertility among brothers of subfertile men may be caused by autosomal recessive inheritance, as proposed in the study by Lilford *et al.,* (1994). The prevalence of consanguineous marriages among parents of our subfertile men is 5.5%, which may also point to an autosomal recessive inheritance. Alternatively, sex limited autosomal dominant or X-linked inheritance may also be involved in male subfertility and may as well explain the increased frequency of male subfertility among maternal uncles. The finding that there is a higher prevalence of male factor subfertility among maternal uncles than among paternal uncles contributes to this hypothesis. This is not in line with the results of (Lilford *et al.,* 1994; Meschede *et al.,* 2000). It should be noted here that during the study the subfertile men reported a total of 15 nephews, all sons of maternal aunts, with male fertility problems. As there were no direct questions about subfertility among cousins we do not know any numbers for the control group. Therefore conclusions cannot be drawn at this moment.

Our results can be influenced by diverse factors, which contradict the hypothesis on the familial occurrence of male subfertility. Subfertile men may be better informed on fertility problems in their families than men from the control group, causing recall bias. This may explain the differences found in the prevalence of fertility problems between brothers and maternal uncles of subfertile men and their controls. Therefore we also compared the prevalence of fertility problems among family members with the prevalence of fertility problems among responders of the control group, which is in the same range as earlier reports and indicates the population risk (Beurskens *et al.,* 1985; van Golde unpublished data). No statistically significant differences could be found comparing the prevalence of fertility problems among brothers of subfertile men to the prevalence of fertility problems among responders of the control group, but there still is an OR of 2.1 (95%CI; 0.8-5.8). To search for possible new genetic causes of male subfertility we should exclude all known genetic causes in the analysis of the family history. No significant differences can be found between relatives of subfertile men and controls when excluding these known genetic causes.

On the other hand the familial occurrence of male subfertility may even be higher than found in this study considering the following factors. The calculated Odds ratios among brothers and maternal uncles may even be higher, if fertility problems among brothers and maternal uncles of subfertile men using a family history were also underestimated. Non-paternity is another factor that may lead to underestimation of the calculated odds ratios. If male subfertility has a familial occurrence then the opportunities for conception through another man are greatest among partners of subfertile men in these families. Finally, men may have poor sperm results on sperm analysis but some of them may still father children.
This study also investigated the phenotypic characteristics of subfertile men. The clinical and genetic data revealed the normal frequencies as mentioned in the literature. A new finding is that subfertile men with a positive family history more often have normal serum concentrations of FSH and LH. This phenomenon is also observed in men with Y microdeletions in the AZF c region (Kremer *et al.,* 1997).
From the current study we can conclude that male subfertility has a familial occurrence, although different kinds of bias may have influenced the results. Further basic and clinical research will contribute to our knowledge of genetic aetiology and the clinical approach in cases of male subfertility.

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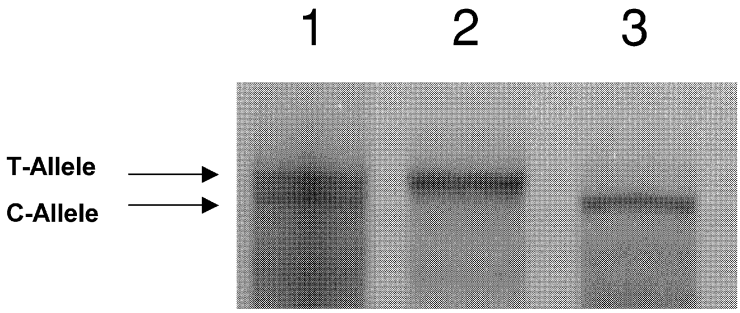
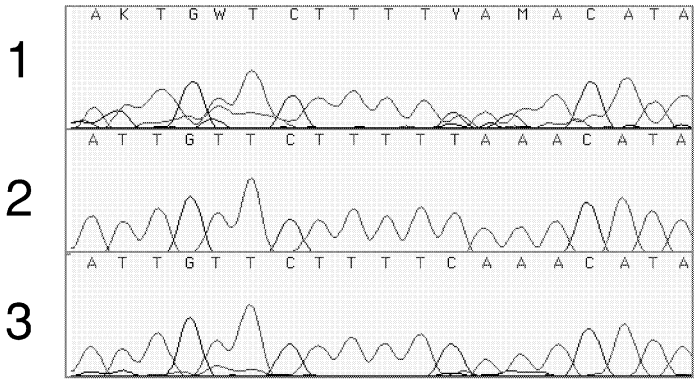
Part II

Molecular genetic studies in male subfertility





Chapter 3

Familial oligo-astheno-teratozoospermia: Evidence for autosomal dominant inheritance with sex limited expression

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Abstract

Objective: Severe oligo- astheno- teratozoospermia (OAT) is a rare disorder occurring in approximately 0.3% of all males. The familial occurrence of severe OAT in a male and five male relatives related through their mothers is reported. In addition we performed an X chromosome linkage study.

Design: Case report

Setting: University medical centre.

Patient(s): six affected family members.

Intervention(s): Blood and semen samples were collected from all affected males and some of their healthy male relatives

Main outcome measure(s): Pedigree analysis and exclusion of X-linked disorder.

Results: Familial non-syndromic male factor infertility

Conclusion(s): The presented family suggests that an autosomal dominant trait of male infertility with sex limited expression exists.

Introduction

Severe oligo- astheno- teratozoospermia (OAT; < 1 x 10^6 spermatozoa /ml with progressive motility per ejaculate) or azoospermia is a rare disorder in otherwise healthy men. The estimated prevalence is approximately 0.3% in the male population (Kremer *et al.,* 1997). In most cases with severely impaired semen parameters the aetiology remains obscure and it has been suggested that genetic factors may play an important role (De Kretser, 1997). The current lack of knowledge regarding genetic factors involved in spermatogenesis is a source of concern, since the transmission of these unknown factors to the offspring by ICSI is real. Large-scale family studies in defined populations that might reveal these genetic factors are scarce (Budde *et al.,* 1984; Lilford *et al.,* 1994). These studies in addition with the case reports of infertile brothers all suggest that a substantive part of undiagnosed male infertility cases might arise from autosomal recessive mutations (Budde *et al.,* 1984; Lilford *et al.,* 1994; Meschede *et al.,* 2000). Today, little is known about autosomal dominant or X-linked factors involved in non-syndromic male infertility.
We describe here a family in which idiopathic severe OAT or azoospermia is present in several third degree relatives, the mothers of these males being sisters (see figure 1). We are not aware of publications concerning similar large families with idiopathic non-syndromic male infertility.

Materials and Methods

The proband of the family was identified at the fertility clinic of the University Medical Centre Nijmegen because of primary infertility. The family history was obtained by interview and family members were invited for evaluation of their fertility status, including andrological examination, karyotyping and screening for microdeletions of the Y chromosome (Hoefsloot *et al.,* 1997). In addition blood samples were taken, after obtaining informed consent, for molecular research purposes.
DNA from peripheral lymphocytes was isolated according to the procedure of Miller *et al.*, (1988). The family was screened for linkage with the X-chromosomal markers of the ABI PRISM Linkage Mapping Set, version 2 (PE Biosystems). These markers were selected from the Généthon human linkage map (Dib *et al.,* 1996) and are spaced every 10 cM, on average.

This study was performed with the approval of the institutional human investigation review board.

Results

Case 1

The proband (III-5, see figure 1), now aged 33, is the third child of non consanguineous healthy Dutch parents. He has two healthy brothers. At 5 years of age he had a torsion of a testicle for which he was surgical treated. The proband has epilepsy for which he uses anti-epileptic medicine. He and his wife presented at our clinic with a history of primary infertility of 2 years. His infertility was evaluated at the age of 30. Semen analysis revealed severe OAT (3.0 x 10^6 spermatozoa/ml) while andrological examination showed no additional abnormalities The hormonal profile is within normal range. He has a normal karyotype, while Y chromosomal microdeletion screening did not reveal abnormalities. The family history was positive for male infertility.

Case 2

III-6 is a third degree relative of the probandus (III-7) and he is 45 years old. His parents are non-consanguineous and he has one sister, three healthy brothers and a fourth brother who also experienced infertility. III-6 and his 45 year old wife remained childless involentary. They first attended our clinic in 1980. Semen parameters were impaired during all medical examinations until today (0.6 x 10^6 spermatozoa/ml). The andrologic history shows a surgical correction of a varicocele. Andrological examination including, hormonal profile, karyotyping and screening for microdeletions of the Y chromosome did not reveal additional abnormalities.

Case 3

This 43-year-old man (III-9) is the brother of case 2. He and his 43-year-old partner had also experienced primary infertility for years. Andrologic history reveals no abnormalities except for the severely impaired semen parameters. They remained involuntary childless, which they experienced as a grave burden.

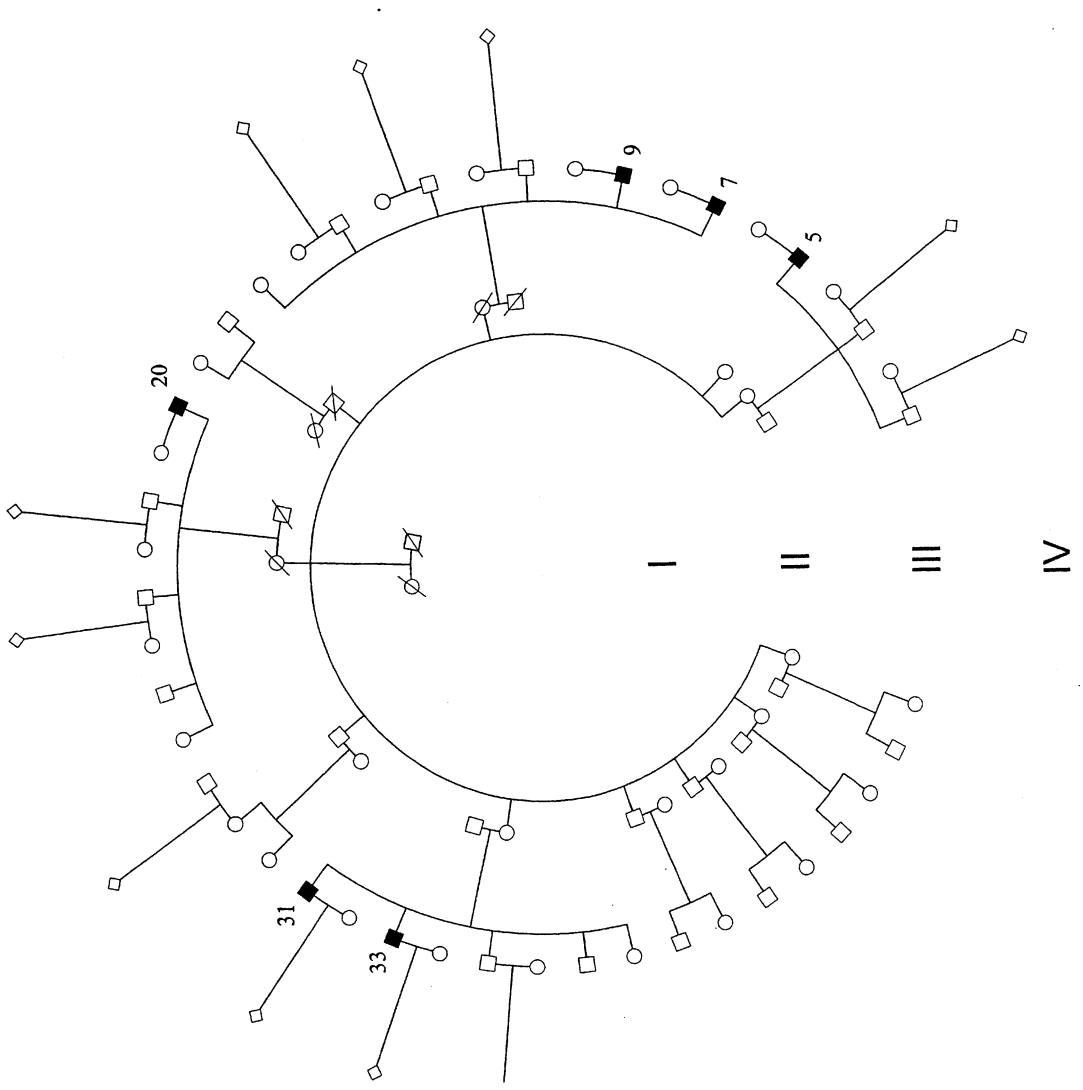

Figure 1. Pedigree of the probandus (III-5), the filled quadrants represent males with male factor infertility

A recent semen analysis confirmed the earlier results (0.1 x 10^6 spermatozoa/ml) and the karyotype was 46,XY. Screening for Y chromosome microdeltions revealed an AZFc deletion.

Case 4

Case 4 is a 43-year-old third degree relative (III-20) of case 1. His parents are non-consanguineous. He has three healthy brothers and a sister. He and his 42-year-old partner experienced primary infertility for some years. Their medical doctor informed them that it would not be possible for them to get any children.
The andrologic history reveals a surgical correction of an inguinal hernia at 5 years of age. Andrological investigation showed a non-obstructive azoospermia: both testicles have a small volume, the ejaculate has a normal volume and the serum FSH level is high. The karyotype was 46,XY and screening for microdeletions of the Y chromosome did not reveal abnormalities.

Case 5

He also is a third degree relative of case 1 (III-31), aged 44 years. His parents are non-consanguineous. He has four sisters, two unaffected brothers and one brother with infertility. He had surgical correction of a varicocele but the semen parameters remained impaired (10 x 10^6 spermatozoa/ml). Andrologic history and examination of this primary infertile male shows no additional abnormalities. The karyotype is 46,XY and no microdeletions of the Y chromosome could be detected.

Case 6

The brother of case 5, is a 43-year-old man (III-33) with severely impaired sperm parameters. The andrologic history shows torsion of the right testicle, impaired semen parameters (0.1 x 10^6 spermatozoa/ml) and an increased FSH serum concentration. The karyotype is 46,XY and there are no microdeletions of the Y chromosome.

Relative III-24 and his wife experienced primary infertility for 8 years because of a presumed female factor. After IVF treatment they conceived a female twin, now aged 5 years. The semen parameters at the time of the IVF procedures were reported to be within the normal range. All other male relatives in this family did not reported fertility disorders. Some of the male relatives who did not report a fertility disorder agreed to have an

andrological evaluation (individuals III-1, III-11, III-13, III-15, III-24 and III-35, see figure 1). The examination of these healthy fertile males did not reveal abnormalities except for a variococele in III-35.

A routine fertility screening of the partners of the affected males did not reveal abnormalities. Also, none of the female family members has reported fertility disorders. In addition, no development disorders or malformations have been reported in this family.

DNA analysis

Screening with markers of the X chromosome revealed that none of the markers, selected from the Généthon human linkage map (Dib *et al.,* 1996) and spaced every 10 cM on average, segregated with the disease in the family.

Discussion

The presented family shows a clustering of male relatives with severe OAT or azoospermia. The heterogeneity of the andrological related diagnosis in this family might indicate that the fertility problems in this family are not due to single disorder. However, excluding the one male with an AZFc microdeletion, it is unlikely that this clustering of males with severe OAT or azoospermia can be explained by chance alone (probability $2x\ 10^{-6}$). The performed molecular analysis with X linked markers shows that the impaired spermatogenesis in this pedigree is not due to an X-linked disorder. Therefore, this pedigree suggests for the existence of an autosomal dominant trait for a disorder with severely impaired spermatogenesis. Although, a multifactorial aetiology cannot be excluded completely.

The reports on familial non-syndromic male factor infertility suggest that inheritable male factor infertility concern autosomal recessive disorders (Budde *et al.,* 1984; Lilford *et al.,* 1994; Meschede *et al.,* 2000). However, it seems likely that autosomal dominant or X linked non-syndromic male factor infertility also exists. EEC syndrome, myotonic dystrophy, Kallmann syndrome and Aarskog syndrome are examples of syndromes associated with male factor infertility and these are disorders with an autosomal dominant or X-linked inheritance. Chaganti and German reported in 1979 on a non-syndromic infertile male with an infertile maternal uncle and an infertile son of a maternal sister. They postulated that a gene for meiotic disturbance is segregating in their family. We observed in our cohort of ICSI males some small families with brothers and/or maternal uncles

and/or sons of maternal sisters with non-syndromic male factor infertility (data not published). In addition, we noted the large family with non-syndromic male factor infertility, which is presented here.

In conclusion, the presented family may show an autosomal dominant factor of male infertility, with sex limited expression. Further molecular research, especially a genome wide screen for linkage, in this family and others may elucidate the gene(s) involved. In our view the family history of infertile males should not be limited to first degree relatives but should also include questions concerning the fertility status of all second and third degree relatives.

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Chapter 4

DAZLA: An important candidate gene in male subfertility?

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Abstract

Purpose: To study the role of the autosomal candidate gene *DAZLA* (*D*eleted in *AZ*oospermia *L*ike *A*utosome) in male subfertility.

Methods: We reviewed clinical data of subfertile men with oligozoospermia or azoospermia, mostly candidates for intracytoplasmic sperm injection (ICSI). Mutation detection was performed using polymerase chain reaction followed by single strand conformation polymorphism analysis. All shifted bands were analysed by sequencing.

Results: We searched for mutations in 44 subfertile men. Nine subfertile men were included, because family history showed that their brothers also faced fertility problems. In these men a possible autosomal gene defect may contribute to their fertility problem.

No mutations were found, except for two polymorphisms in intron 4 and 5.

Conclusion: At this moment it does not seem relevant to search for possible mutations in the *DAZLA* gene in clinical practice.

Introduction

Genetic factors may play an important role in the aetiology of severe oligozoospermia or azoospermia causing male subfertility. Chromosomal aberrations, microdeletions of the Y-chromosome and cystic fibrosis trans-membrane receptor gene (CFTR) mutations have been described in men with severe subfertility (Tuerlings *et al.,* 1997). Of particular interest is a study from Lilford *et al.,* (1994) showing a possible autosomal recessive mode of inheritance in male subfertility.

The *DAZLA* (*D*eleted in *AZ*oospermia *L*ike *A*utosomal) gene on human chromosome 3 shares a high degree of homology with the DAZ (*D*eleted in *AZ*oospermia) gene, which is sometimes deleted in men with azoospermia or severe oligozoospermia. It is suggested that the DAZ genes arose from the transposition of *DAZLA* to the Y-chromosome followed by repeated amplification and pruning (Saxena *et al.,* 1996). Its testis specific expression and its homology to DAZ support the role of *DAZLA* in spermatogenesis. Disruption of the *DAZLA* gene in mice leads to loss of germ cells in both ovary and testis and absence of gamete production, demonstrating that *DAZLA* is essential for the differentiation of germ cells in mice (Ruggiu *et al.,* 1997).

It might be argued that mutations in the human *DAZLA* gene are responsible for some of the (familial) cases of male subfertility. Since the genomic structure of *DAZLA* is known mutation detection is possible, but to our knowledge no such studies have been reported till now. In this study, we searched for mutations in the *DAZLA* gene in severe subfertile men, using Single Strand Confirmation Polymorphism (SSCP) and sequencing techniques.

Materials and Methods

Patient selection

The study population was selected from a group of 300 intra cytoplasmic sperm injection (ICSI) men attending our fertility clinic since 1996, with abnormal sperm parameters varying from severe oligoasthenoteratozoospermia (OAT) to azoospermia.

Semen analysis was performed according to World Health Organization (WHO, 1992) guidelines. When more than one semen analysis was available, only the data of the first sample were used. In our centre, oligoasthenozoospermic men are candidates for ICSI if

their ejaculate contains less than 1.0×10^6 propulsive spermatozoa. Andrologic history and examination, family history and hormone measurements were performed. In addition, all men were offered chromosome analysis and screening for microdeletions of the AZFa, b and c region of the Y chromosome (Kremer *et al.,* 1997). In cases of Congenital Bilateral Absence of the Vas Deferens (CBAVD) the cystic fibrosis trans-membrane receptor gene was screened for mutations.

Men with a documented cause for their subfertility such as previous sterilization, testicular malignancy, CBAVD, chromosomal abnormality or Y chromosome microdeletion were excluded from further analysis.

In this study we selected all men whose family history showed that a brother was also facing fertility problems. The remaining men included in this study were randomly selected. The local institutional review board approved this study.

Mutation analysis

Genomic DNA was isolated from peripheral blood as described previously (Miller *et al.,* 1988). The *DAZLA* gene has 11 exons. For exons 2-11 PCR fragments were generated. Primers were designed using the published sequence of the DAZLA gene, GB: U77467-U77476 (Table I). Exon 1, containing only the first 3 coding basepairs was not analysed. The size of the PCR fragments varied between 241 and 399 bp. Amplified fragments were analysed for single strand confirmation polymorphism (SSCP), using GeneGel Excel 12.5/24 Kit (Pharmacia Biotech AB, Roosendaal). Fragments were stained with the DNA Silver Staining Kit (Pharmacia Biotech AB, Roosendaal).

PCR fragments generating a shifted band were analysed by direct sequencing using the Big dye Terminator cycle sequencing (PE Biosystems, Foster City). The sequence reactions were run and analysed using an automated sequencer, 3700 (PE Biosystems, Foster City).

Results

We selected 44 men; 9 out of these 44 men were included because the family history showed that their brothers also faced fertility problems, the other 35 men were randomly selected.

The main clinical characteristics, testis volume, semen analysis, and hormone measurements of these males are shown in Table II. Thirty-seven men had oligoasthenoteratozoospermia, three men had asthenozoospermia and four men had azoospermia. The most frequently encountered abnormality was cryptorchidism (n = 6), followed by history of male adnexitis (n = 4), inguinal hernia (n = 3), and varicocele (n = 2). Clinical characteristics of the men with a brother facing fertility problems did not differ from the other men.

A total of 25 aberrant shifts were identified by SSCP in the 44 men screened for mutations in the *DAZLA* gene. Sequence analysis revealed no alteration in the coding area and the splice sites when compared to the genomic structure (Chai *et al.,* 1997).

Shifts in exon 4 and 5 were shown to be neutral polymorphisms. At 30 bp upstream of exon 4, a variant was found (A/C) and at 28 bp upstream exon 5 another variant was found (T/C; Figure 1). In a control group of 20 males the same distribution of these variants was detected (not shown).

Table I. PCR primers for the *DAZLA* gene

Exon	Primers (5'-3')	Fragment length in bp
Exon 2	F: TGTAAAACGACGGCCAGTCTGAGCCTGAACTAACTTAGAATG R: CAGGAAACAGCTATGACCGTTTGTAACAGGGCCCAAATC	377 bp
Exon 3	F: TGTAAAACGACGGCCAGTTAAAATTAAAATTTTTGAATGCTG R: CAGGAAACAGCTATGACCAGAGCTGGCAATAAACTTTTATCC	332 bp
Exon 4	F: TGTAAAACGACGGCCAGTATTTTAGTCATGATCACTTCCG R: CAGGAAACAGCTATGACCTGTAGTTCATGAACCTAGGTGC	371 bp
Exon 5	F: TGTAAAACGACGGCCAGTTCCAAGTCTTGGAAGTAAAGAC R: CAGGAAACAGCTATGACCGATAAGCACCTTTTTGAAAAGC	268 bp
Exon 6	F: TGTAAAACGACGGCCAGTCAATCAGGAAACAAAATTTATG R: CAGGAAACAGCTATGACCCCACAGAAGGTACGATGACTAC	353 bp
Exon 7	F: TGTAAAACGACGGCCAGTTTTTCATATTTTTGTTATATTGGG R: CAGGAAACAGCTATGACCATGACAAACCATTCAGACAATTTG	285 bp
Exon 8	F: TGTAAAACGACGGCCAGTTATTATAACAACAAAGGAGCCAGC R: CAGGAAACAGCTATGACCTAGGCATATATGACATGGAAAACG	301 bp
Exon 9	F: TGTAAAACGACGGCCAGTTTAGCTTTTTGAAGAATAAGTGGC R: CAGGAAACAGCTATGACCTTTCTTTTACTATTTGGTCAAGCC	399 bp
Exon 10	F: TGTAAAACGACGGCCAGTGAAAGAGTGGTCTTTACATTAGTG R: CAGGAAACAGCTATGACCAACTACATTATGTCAAGGTTCAGC	363 bp
Exon 11	F: TGTAAAACGACGGCCAGTAGAAATTTTCAGTAAAGTAAAAGG R: CAGGAAACAGCTATGACCAGCTTAATATTCAAAACCAGCAAC	246 bp

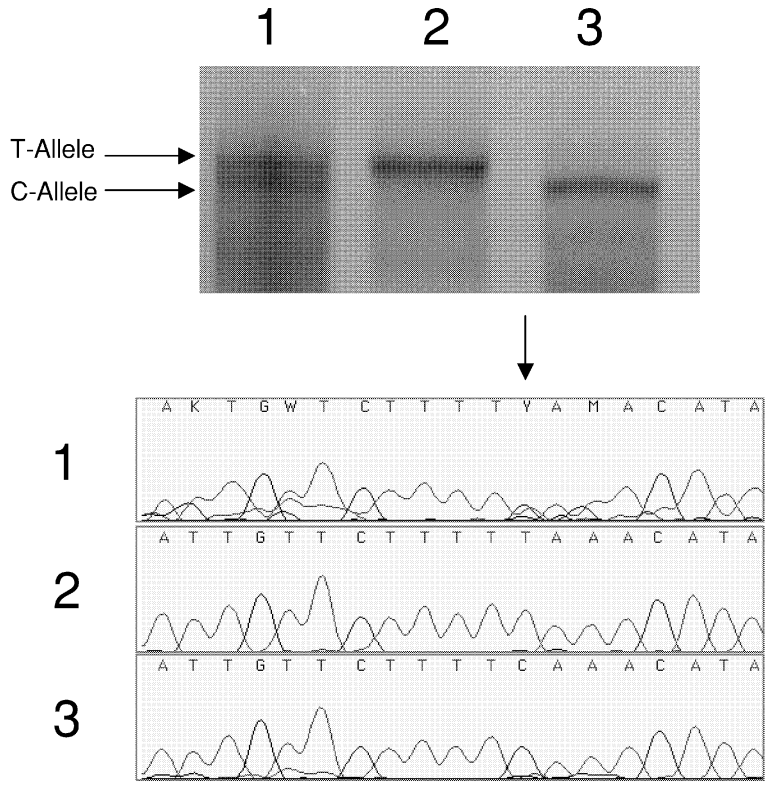

Figure 1. SSCP and sequence analysis showing the polymorphism found in intron 5 twenty-eight base pairs upstream of exon 5 (IVS5-28T/C). Lane 1-3: patient DNA.

Table II. Testis volume, semen analysis and hormone measurements of the 44 males screened for mutations in *DAZLA*

Patients (n = 44)	Testis Volume (ml)	Sperm concn ($\times 10^6$ ml)	Motility (% propulsive)	FSH [a]	LH [b]	Testosterone
Local reference range	***>15***	***>20***	***>50***	***2.0 - 7.5***	***1.8 - 9.5***	***11 - 45***
OAT [c] (n = 37)	15.3 ± 0.7	3.9 ± 3.5	27.6 ± 17.9	7.3 ± 0.7	3.8 ± 0.3	15.8 ± 1.0
Asthenozoospermia (n = 3)	18.3 ± 2.9	51.7 ± 22.5	0	5.0 ± 2.7	2.6 ± 0.7	17.0 ± 4.6
Azoospermia (n = 4)	12.3 ± 1.0	0	0	14.3 ± 5.4	5.7 ± 0.8	18.0 ± 0.9

Note. Values are presented as Mean ± SD
[a]FSH, follicle stimulating hormone; [b]LH, luteinizing hormone; [c]OAT, oligoasthenoteratozoospermia

Discussion

In 40-60% of cases, the aetiology of male subfertility remains unknown and has to be classified as idiopathic (de Kretser, 1997). It has been suggested that in cases of idiopathic male subfertility there may be a genetic origin (Tuerlings *et al.,* 1997).
Some of these genetic factors are microdeletions of the Y chromosome and the CFTR gene.
The frequency of Y chromosomal DAZ inclusive deletions occur in a frequency of 13% in azoospermic and 7% in severely oligospermic men, and 1 to 29% in subfertile men (Kremer *et al.,* 1997; Reijo, *et al.,* 1995; Silber *et al.,* 1998). This frequency is dependent on the definition of male subfertility and on the choice of Sequence Tagged Sites used for screening (Simoni *et al.,* 1999). There are more candidate genes on the Y chromosome, which could play a role in male factor subfertility (Schnieders *et al.,* 1996; Lahn and Page, 1997; Kent First *et al.,* 1999).
Recently, an azoospermic man with a de novo point mutation in the Y chromosomal gene USP9Y has been described (Sun *et al.,* 1999). This study shows the importance of the deletion of this gene as seen subfertile men with an AZFa deletion. However, given the low frequency reported in this study (1/576 men) routine screening of this gene in ICSI candidates probably has no clinical relevance.
Another common monogenic disorder present in subfertile men with CBAVD is Cystic Fibrosis (CF), caused by mutations in the CFTR gene. The frequency of CBAVD in subfertile men is low and the frequencies of the CFTR mutations among subfertile men do not differ from normal frequency (Tuerlings *et al.,* 1998a).
Furthermore, an inactivating point mutation of the FSH receptor gene has been reported in some men with elevated serum FSH concentrations and abnormal sperm parameters (Tapanainen *et al.,* 1997). In a study of 28 men with a high level of FSH, no mutations were found in the FSH receptor gene (Tuerlings *et al.,* 1998b).
Information on the genetic basis of male subfertility is important. The cause of male subfertility is often unknown and in this group of idiopathic male subfertility, genetic factors could be involved. *DAZLA* may be one of the genes important in the pathogenesis of male subfertility.
To come to an aetiological diagnosis rather than a purely descriptive diagnosis is not only more satisfactory to the doctor, it will also be beneficial to patients. Coping with subfertility can be facilitated by a specific diagnosis and at risk relatives may be recognised earlier

(Meschede *et al.,* 1995; In't Veld *et al.,* 1997). Only by the identification of the aetiology of male subfertility progress can be made towards a better therapy and prevention. In this way genetic risk for the offspring could be correctly evaluated and dealt with, especially nowadays since intracytoplasmic sperm injection circumvents a part of the natural selection mechanism.

In this study we searched for mutations in the *DAZLA* gene in male subfertility. Disruption of the *DAZLA* gene in mice leads to loss of germ cells and absence of gamete production (Ruggiu *et al*., 1997) resulting in azoospermia. Mutations in the human *DAZLA* gene may lead to oligoasthenoteratozoospermia in men comparable to the effect of the deletions of the Y chromosomal DAZ gene, and may be responsible for some of the familial cases of male subfertility (Lilford *et al.,* 1994). No mutations were found, but two neutral polymorphisms were identified in the introns 4 and 5. It is not likely that *DAZLA* mutations have been missed in a majority of cases, although SSCP detects only between 80%-90% of all possible mutations and we did not analyse exon 1, containing only the first codon. Besides that with a study group consisting of 44 subfertile men statistical analyses shows that there is 95% certainty that the possible mutation frequency in the DAZLA gene is lower than 7%. Screening a larger group of patients, like has been performed for the USP9Y gene (Sun *et al.,* 1999), might still result in finding a pathogenic mutation.

Our data suggest that the *DAZLA* gene is not a major contributor to male subfertility. At this moment routine screening in ICSI men for possible mutations in the *DAZLA* gene does not seem relevant in clinical practice.

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Chapter 5

Is increased CAG repeat length in the androgen receptor gene a risk factor for male subfertility?

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Abstract

Purpose: Increased length of the CAG repeat in the androgen receptor gene may be related to male subfertility. Expansion to 38-62 CAG repeats leads to the neurodegenerative disorder with male infertility called Kennedy's disease. Recently it was suggested that slight expansion is related to male subfertility. In this study we investigated the association of male subfertility with the length of CAG repeats in the androgen receptor.

Materials: CAG repeat length in the androgen receptor gene was investigated in 75 subfertile men, who were mainly candidates for intracytoplasmic sperm injection. Sperm parameters varied from azoospermia to severe oligoasthenoteratozoospermia. The control group consisted of 70 men, who predominantly had bladder cancer. DNA was isolated from peripheral blood and genotyping was performed with polymerase chain reaction based methods.

Results: No statistically significant difference in the mean length of the CAG repeat plus or minus standard deviation was noted in subfertile men and controls (21.7 ± 3.4 and 22.2 ± 3.1). The length of the CAG repeat in the androgen receptor was not related to the degree of impaired spermatogenesis or clinical characteristics of the subfertile men.

Conclusion: Increased length of CAG repeats in the androgen receptor gene is not a risk factor for male subfertility.

Introduction

In the Netherlands 1/7 couples do not achieve pregnancy within 1 year of active sexual intercourse and at the end of their reproductive life span 2% to 7% remain childless (Beurskens *et al.,* 1995). Subfertility may have various causes in either partner. According to the World Health Organisation (WHO, 1992), in 20% of all cases the problem is predominantly male and in 38% it is predominantly female. To our knowledge the causes of defective spermatogenesis often remain unknown to date. Recently much attention has been given to genetic causes of male subfertility, such as chromosomal aberrations (Tuerlings *et al.,* 1998), microdeletions of the Y-chromosome (Kremer *et al.,* 1997) and other possible monogenetic causes. This attention is stimulated by the concern of potential transmission of genetic defects to offspring via intracytoplasmic sperm injection.

Androgen has a critical role in regulating spermatogenesis. It acts through the androgen receptor, which is encoded by the androgen receptor gene. The androgen receptor is a member of the steroid receptor superfamily. After androgen is bound to the cytosolic androgen receptor, the receptor is activated. This activated androgen-androgen receptor complex translocates to the nucleus and binds to androgen-responsive elements, leading to regulation of transcription processes of downstream androgen dependent genes (Evans, 1988; Beato, 1989). It has been well documented that androgens acting via the androgen receptor in the Sertoli's cells cause the stimulation and maintenance of spermatogenesis. Moreover, suppression of endogenous androgen production has been used as a type of male contraception (Remy *et al.,* 1996). In contrast, administration of a sufficient dose of testosterone has been shown to maintain complete spermatogenesis in hypophysectomized animals (Buhl *et al.,* 1982).

The androgen receptor gene (*AR*) is a candidate gene in male subfertility. Most patients with idiopathic male subfertility have normal serum levels of androgen, implying that defects in the *AR* may be involved in the aetiology of this condition. In 1979, Aiman *et al.* postulated that defects in the *AR* gene may lead to partial or complete spermatogenic failure.

This *AR* gene, which is located on the long arm of the X chromosome at Xq11-12, contains a polymorphic CAG repeat sequence in exon 1 that encodes a series of glutamine residues in the region of the androgen receptor protein associated with DNA transcription (Brown *et al.,* 1989). In vitro, the length of the CAG repeats inversely correlates with *AR* transcriptional activity (Kazemi *et al.,* 1995). A relatively short CAG

repeat is associated with an increased risk of prostate cancer, which is an androgen dependent tumor (Giovannucci *et al.,* 1997), whereas a length of 38-62 CAG repeats leads to Kennedy's disease, a condition associated with bulbar muscular atrophy, low virilization and depressed spermatogenesis (Nagashima *et al.,* 1988; La Spada *et al.*, 1991). Tut *et al.,* (1997) reported that patients with 26 or more CAG repeats in the *AR* gene were at more than a 4-fold increased risk for impaired spermatogenesis. These findings indicate that some cases of male subfertility may be related to a high but normal range of 26-38 CAG repeats in the *AR* gene.

Several groups reported conflicting results on the role of the CAG repeat length in the *AR* gene in cases of male subfertility (Tut *et al.,* 1997; Yoshida *et al.,* 1999; Downsing *et al.*, 1999; Giwercman *et al.,* 1998; Dadze *et al.,* 2000). We investigated the hypothetical association of expansion of the CAG repeat of the *AR* gene with clinical characteristics among a group of Dutch subfertile men.

Materials and Methods

The study population consisted of 75 men who were mainly candidates for intracytoplasmic sperm injection, and who were randomly selected from a group of 253 who attended our fertility clinic since 1998. Sperm parameters varied from azoospermia to severe oligoasthenoteratozoospermia.

In our clinic subfertile men are candidates for intracytoplasmic sperm injection when their ejaculate contains less than 1.0×10^6 spermatozoa with propulsive motility (WHO, 1992). Andrologic history and examination were done and hormones were measured. In addition, all men were offered chromosome analysis and screening for microdeletions of the AZFa, b and c region of the Y chromosome (Hoefsloot *et al.,* 1997). In cases of Congenital Bilateral Absence of the Vas Deferens (CBAVD) the cystic fibrosis trans-membrane receptor gene was screened for mutations.

In this study we excluded men with previous sterilisation, testicular malignancy, CBAVD, chromosomal abnormality or Y chromosome microdeletion. The patients signed an informed consent form for the use of DNA in anonymous studies of genetics and male subfertility. The local institutional review board approved this study.

The control group consisted of 70 men who presented at the outpatient clinic of the department of urology between March and May 1997. They were asked to participate in a

study of the role of gene-environment interactions in urological disease. The group included men with diseases not known to be related to subfertility or to polymorphism in the androgen receptor gene, including bladder cancer in 61, erectile dysfunction in 3, kidney stones in 3 and vasectomy in 3. Patients with bladder cancer were over represented in this control group because they participated in another simultaneous study on gene-environment interactions. Consequently, their data were already available.

After informed consent was obtained study participants were asked to complete a questionnaire and provide a blood sample. The questionnaire included questions on demographic items, smoking, diet and drinking habits, profession and previous diseases. For CAG repeat analysis genomic DNA was isolated from peripheral blood, as described previously (Miller *et al.,* 1988).

The number of CAG-repeats in the *AR* gene polymorphism, was determined by polymerase chain reaction using the primers sense (5'-AGA GGC CGC GAG CGC AGC ACC TC-3') and antisense (5'-GCT GTG AAG GTT GCT GTT CCT CAT-3'). α-[^{32}P]dATP was incorporated as a radioactive label. We performed denaturation at 95ºC for 40 seconds, annealing at 66ºC for 40 seconds and elongation at 72ºC for another 40 seconds. After 35 cycles elongation was performed for another 10 minutes at 72ºC. Samples were run on a 5% denaturating polyacrylamide gel for 3 hours at 100W. Gels were transferred to Whatmann 3-mm filter paper, dried, and exposed to X-ray film. Control samples were sequenced and used as standards to determine the size of the sample alleles under investigation.

Statistical analysis was performed using commercially available software. The mean plus or minus standard deviations was calculated and compared by the t-tests. Spearman's correlation coefficients were calculated to analyse correlations of the length of the CAG repeat with clinical parameters.

Power analysis was based on data from a previous study of the AR gene polymorphism (Bousema *et al.,* 2000). In that study controls appeared to have a mean repeat length plus or minus standard deviation of 21.9 ± 2.9. Similar values are reported in the literature. On power analysis we assumed a standard deviation of 3. With 70 cases and 70 controls we detected a difference in repeat length of 1.4 with a power of 80%.

Results

The Table I lists main clinical characteristics of the 75 subfertile men, including testis volume, semen analysis and hormone measurements. A total of 61 men had oligoasthenoteratozoospermia, 4 mainly had an asthenozoospermia and 10 had azoospermia. The most common clinical abnormality was cryptorchidism in 22 cases, followed by a history of male adnexitis in 6, surgical correction of inguinal hernia in 9 and varicocele in 14. None of the study participants showed a sign of neuromuscular disease. Participants were predominantly white. Neither patients nor controls included men of Chinese or Indian ancestry, who are known to have a longer CAG repeat length (Sartor *et al.,* 1999).

No statistically significant difference in the mean length of the CAG repeat was detected in subfertile men and controls (21.7 ± SD 3.4 and 22.2 ± 3.1, respectively). Figure 1 shows the distribution of CAG repeats in both groups. We did not identify a subgroup of subfertile men with an increased number of CAG repeats. Also, the number CAG repeats did not correlate with the number, the motility or the morphology of sperm, or with follicle-stimulating hormone, the luteinizing hormone or testosterone.

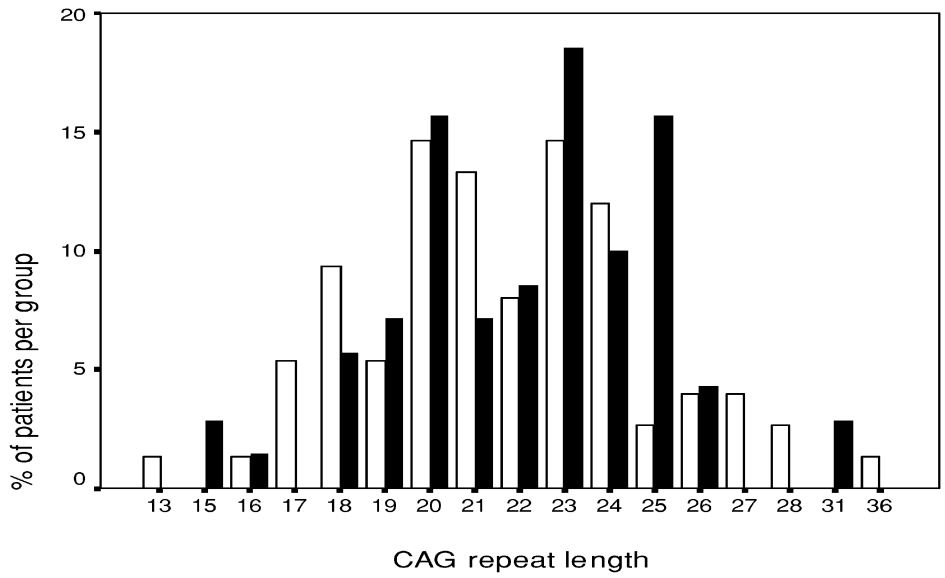

Figure 1. Distribution of CAG repeat sizes in exon 1 of androgen receptor gene in subfertile men (white bars) and controls (black bars).

Table I. Testis volume, semen analysis and hormone measurements of 75 subfertile men studied for CAG repeat length of the androgene receptor gene

Patients (n = 44)	**Testis Volume (ml)**	**Sperm concn ($\times 10^6$ ml)**	**Motility (% propulsive)**	**FSH [a]**	**LH [b]**	**Testosterone**
Local reference range	*>15*	*>20*	*>50*	*2.0 - 7.5*	*1.8 - 9.5*	*11 - 45*
OAT [c]	16.4 ± 4.3	2.6 ± 3.0	23.1 ± 14.7	8.7 ± 5.3	4.7 ± 2.3	18.4 ± 8.3
(n = 61)						
Asthenozoospermia	17.5 ± 5.0	28.8 ± 11.8	6.5 ± 6.0	9.1 ± 3.1	4.8 ± 0.6	17.5 ± 5.0
(n = 4)						
Azoospermia	12.7 ± 4.2	0	0	19.4 ± 11.4	7.3 ± 3.9	17.1 ± 5.2
(n = 10)						

Values are presented as Mean ± SD
[a]FSH, follicle stimulating hormone; [b]LH, luteinizing hormone; [c]OAT, oligoasthenoteratozoospermia

Discussion

Our findings imply that male subfertility is not related to a high but normal range of 26-38 CAG repeats in the *AR* gene. This result is in line with Swedish (Giwercman *et al.,* 1998) and German (Dadze *et al*., 2000) investigations. However, Tut *et al.,* (1997) reported in vivo and in vitro data that longer CAG repeats correspond with a less androgenic androgen receptor. This finding was corroborated by the work of Yong *et al.,* (1998) and Downsing *et al.,* (1999). Up to 20% of Chinese men presenting with defective spermatogenesis and subfertility had an increased number of CAG repeats (Yong *et al.,* 1998). Confusingly Komori *et al.,* (1999) reported that a decrease in CAG repeats of 16 or less is strongly related to impaired spermatogenesis in infertile Japanese men. In accordance with studies of a predominantly white population our data do not provide any proof of a relationship of the length of the CAG repeat and the degree of impaired spermatogenesis or clinical characteristics of subfertile men. However, a strong conclusion cannot be drawn from our subgroup analysis due to small numbers.

There are several possible explanations for the apparently conflicting data. The ethnic differences in the study populations yielded different findings in the European and Asian studies. It is well documented in the literature that the size of the CAG repeats in the *AR* gene varies in a race specific manner (Sartor *et al.,* 1999). The prevalence of short CAG repeats (22 repeats or less) is high in black Americans (75%) and relatively low in Asians (49%). Another possible explanation is the choice of control group. Our control group mainly comprised patients with bladder cancer. Bladder cancer is normally not considered as an androgen dependent tumour but there have been observations that the inhibition of androgen production in male rats is related to a somewhat lower risk of N-butyl-N-(4-hydroxybutyl)nitrosamine induced bladder cancer (Imada *et al.,* 1997). If it is also true in men, we may have underestimated the hypothesised association. On the other hand, our finding that the relationship of the polymorphism and male subfertility is similar in men with higher and lower sperm counts argues against a strong association. Moreover, the number of CAG repeats is in the same range as in other studies of white populations.

Recently it was reported that the number of CAG repeats is especially increased in men with azoospermia (Yoshida *et al.,* 1999; Mifsud *et al.,* 2001; Casella *et al.,* 2001). It was not confirmed in the 10 men with azoospermia in our study since mean CAG repeat length was 21.9 ± 2.8. However, 1 man in this study has a relatively high number of CAG repeats

(36). The only clinical feature of this man with subfertility was severe asthenozoospermia but to our knowledge this association has not previously been described in the literature. Currently we are not concerned about the possible increased risk of neurodegenerative diseases in children born after ICSI. Recently it was also shown that children born after ICSI have no increased length of CAG repeats in the *AR* (Cram *et al.,* 2000).

Conclusions

Male subfertility represents a heterogeneous aetiological entity. There is a need for more basic research into the causes and diagnostic approach of male subfertility.

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Chapter 6

Can paternal mitochondrial DNA be transmitted to offspring or extraembryonic tissues after intracytoplasmic sperm injection?

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Submitted

Abstract

There is a risk that IntraCytoplasmic Sperm Injection (ICSI) may increase the transmission of mtDNA diseases to children born after this technique. Knowledge of the fate and transmission of paternal mitochondrial DNA is important since mutations in mitochondrial DNA have been described in oligozoospermic males.

We have used an adaptation of solid phase mini-sequencing to exclude the presence of levels of paternal mtDNA in excess of 0.001% in ICSI families. This method is more sensitive than those used in previous studies and is sufficient to detect the likely paternal contribution (about 0.1-0.5% from simple calculations of expected dilution during fertilisation). Using this method we were able to detect less than 0.001% paternal mtDNA in a maternal mtDNA background. No paternal mtDNA was detected in the embryonic (blood or buccal swabs) tissue of children born after ICSI and in mainly extraembryonic tissue (placenta or umbilical cord). In conclusion, we did not detect paternal mtDNA in blood, buccal swabs, placenta or umbilical cord of children born after ICSI. We have found no evidence that ICSI increases the risk of paternal transmission of mtDNA and hence of mtDNA disorders.

Introduction

It has been assumed that human mitochondrial DNA (mtDNA) is essentially maternally inherited because sperm mtDNA is selectively destroyed after fertilisation (Kaneda *et al.,* 1995). MtDNA mutants can cause severe diseases in humans (Suomalainen, 1997) and cytoplasmic male sterility (CMS, or nonfunctional pollen) in higher plants (Young and Hanson, 1987). Hence mitochondrial dysfunction might also be a feature of human male infertility and mutations in mtDNA have been described in subfertile men (Folgero *et al.,* 1993).

Intracytoplasmic Sperm Injection (ICSI) has transformed the treatment of male infertility but has theoretical genetic risks. ICSI is an increasingly common treatment for infertility, particularly for men with low or absent sperm counts, or immotile sperm. Sperm are collected from ejaculates, or are harvested by PESA (percutaneous epidydimal sperm aspiration) or by aspiration/extraction from the testis (TESA, TESE), and a single sperm is injected into an oocyte. In some centres it is used in preference to simple in vitro fertilisation (IVF) due to its high success rate. One of the reasons for this success is that it bypasses a number of stages of natural fertilisation, including zona pellucida penetration and gamete membrane fusion. Major concerns are firstly, that this may also bypass the supposed mechanism (Kaneda *et al.,* 1995) by which paternal mtDNA is removed from the embryo. MtDNA is more likely to suffer free radical-induced damage than nuclear DNA (Yakes and Van Houten, 1997), so sperm that have undergone oxidative stress could deliver defective mtDNA to an oocyte even though the sperm nucleus is unaffected. Similarly, as sperm carry more mtDNA deletions than oocytes (Reynier *et al.* 1998), there is more potential for passing on mtDNA disease. Secondly, it is clear that ICSI removes much of the selection against genes for male infertility (Page *et al.,* 1999; Phillipson *et al.,* 2000; Silber *et al.,* 1998). This is particularly important in the case of mtDNA because it has been implicated in human male infertility (Folgero *et al.,* 1993; Ruiz-Pesini *et al.,* 2000a; Ruiz-Pesini *et al.* 2000b). Transmission of mutated paternal mtDNA via ICSI may thus lead to progressive and debilitating disorders.

In normal individuals the vast majority of mtDNAs are identical (homoplasmy) but in mtDNA disease heteroplasmy (coexistance of mutant and normal mtDNA) is common. Because a point mutation may become homoplasmic and hence the mtDNA founder of a maternal lineages within a single generation (the so-called bottleneck, Blok *et al.,* 1997), small quantities of exogenous mtDNA could have a profound effect. The paternal

component would comprise up to 0.5% of the 100,000 mtDNAs in each oocyte, but could potentially be amplified to a significant population in the progeny because of these unique genetics.

Previous studies have not detected a paternal contribution to the mtDNA in offspring of ICSI pregnancies at levels in excess of 0.01-1% (Danan *et al.,* 1999; Houshmand *et al.,* 1997; Torroni *et al.,* 1998). Furthermore these studies did not investigate extraembryonic tissue. This is important, because mtDNA may have an uneven distribution between different tissues in a single individual (Poulton *et al.,* 1995). Differences in the ratio between wild type and mutant DNA in different tissues, so called heteroplasmy, is well known for pathogenic mtDNA in mitochondrial diseases (Taylor *et al.,* 1997). If paternal mtDNA did survive after ICSI and replicate during development, this might mean that paternal mtDNA could be enriched in some tissues and diluted in others.

We have adapted solid phase minisequencing, a highly sensitive method for quantitating mixed populations of mtDNAs, to exclude levels of down to 0.001% paternal mtDNA. In this study the origin of mtDNA was investigated in placenta, umbilical cord, blood and buccal swabs of children born following ICSI.

Methods

DNA was extracted by standard methods (Sambrook *et al.,* 1989). We obtained mtDNA sequence data for a 400 bp region of the first hypervariable region of the large noncoding region in ICSI parent-child pairs. M13 tagged primers;
TGTAAAACGACGGCCAGTCTCAAATGGGCCTGTCCTTG (forward primer) and CAGGAAACAGCTATGACCTTGATTTCACGGAGGATGGTG (reverse primer) were used to PCR amplify the region from bp 15875 to bp16421 using ABgene PCR Mastermix with 1.5mM $MgCl_2$. 5 pmoles of primer and 1ul of DNA (at approx 50ng/ul) were used in a 50ul reaction volume. Amplifications were performed for 40 cycles (1 min each at 94°C, 55°C, 72°C, with an initial denaturing step of 4 min at 94°C and a final extension of 10 min at 72°C. Sequencing reactions used standard ABI Bigdye terminator chemistry and conditions and primers corresponding to the M13 tags (TGTAAAACGACGGCCAGT and CAGGAAACAGCTATGACC). Reactions were run on an ABI 377XL Prism DNA Sequencer. Polymorphic sequence variants that could be used to distinguish between paternal and maternal mtDNA were identified. This was followed by solid phase mini-

sequencing. This is a highly sensitive method, involving sequence-specific incorporation of radionucleotides in a primer extension assay (see Figure 1). A forward primer AAGTAGCATCCGTACTAT and a 5' biotinylated reverse primer were used to PCR amplify the region of mtDNA bp15800 to bp16417 using the same conditions as for the PCR for sequencing, except that an annealing temperature of 50°C was used. 10ul PCR product was added to 15ul binding buffer (TE with 2M NaCl) and bound to the wells of a Streptavidin coated 96 well plate (ABgene) for 1 hour. Plates were washed 3 times with washing buffer (TE with 1M NaCl). 50ul of a PCR mix was added, denatured for 5 min at 94°C and extended at 50°C for 10 min. The PCR mix contained 1.5mM $MgCl_2$, 0.1U of Taq Polymerase, 5pmoles of a primer corresponding to the 18bp upstream of the polymorphic base change of interest and 27nmoles of a single ^{32}P radiolabelled dNTP corresponding to either the maternal or the paternal sequence. The plate was washed 3 times with washing buffer and 30ul denaturing buffer added (80% formamide, 10mM EDTA (pH 8.0) 1mg/ml xylene cyanol, 1mg/ml bromophenol blue. The plate was heated to 95°C for 5min and transferred to ice. 4ul samples were loaded onto 1mm thick, 8cmx8cm 8% acrylamide 1xTBE, 7M urea gels and electrophoresed for 45 minutes at 180V. Gels were dried under heat and vacuum and exposed to storage phosphor screens for quantitation of radioactivity in a phoshorimager.

Table I. Polymorphic base changes used to identify maternal and paternal mtDNA.

Pregnancy	Polymorphic base change
Pregnancy 1	16296 T- C
Pregnancy 2	16069 T- C
Pregnancy 3	16239 T- C
Pregnancy 4	16069 T- C
Pregnancy 5	16189 T- C
Pregnancy 6	16267 T- C
Pregnancy 7	16298 T- C
Pregnancy 8	16298 T- C
Pregnancy 9	16298 T- C
Pregnancy 10	16256 T- C
Pregnancy 11	16294 T- C

Parental DNA was extracted from blood or buccal swabs, the first hypervariable region of the large noncoding region of mtDNA was amplified by PCR and sequenced using Bigdye cycle sequencing. The polymorphic changes identified were used to design detection primers encompassing the 18bp upstream of the polymorphism

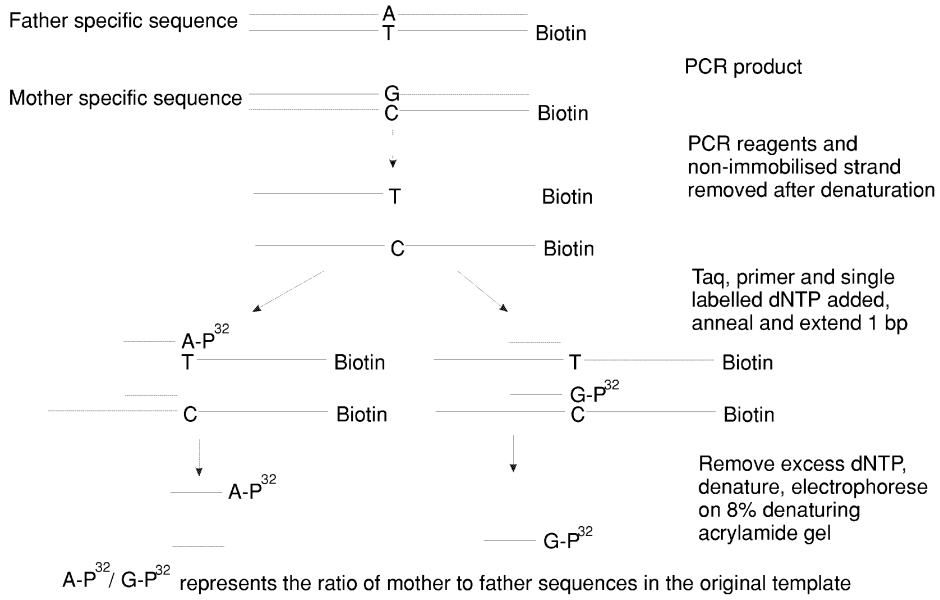

Figure 1. Principles of solid phase mini-sequencing, utilising a G->A base change. The region of interest is amplified by PCR using a biotinylated reverse primer, to allow immobilisation of the product on a streptavidin coated plate. Excess reagents are removed and the plate washed. A single round of PCR is performed using a detection primer corresponding to the sequence immediately upstream of the polymorphism and a radiolabelled dNTP, corresponding to either the paternal or maternal sequence. This allows extension by 1bp if the dNTP matches the sequence present. Separate reactions are performed to detect the 2 different sequences. Excess reagents are removed and the plate washed. The primer is removed by denaturation and electrophoresed on 8% acrylamide TBE/urea gels. The gel is dried and exposed to storage phosphor screens to detect radiolabelled primer.

Results

11 ICSI pregnancies were investigated. These ICSI pregnancies were all derived using ejaculated sperm. DNA was extracted from placenta (3), buccal swabs (3) or placenta, umbilical cord and blood (5). Parental DNA was extracted from either blood or from buccal swabs. Sequence analysis using Bigdye cycle sequencing demonstrated that there were usable mtDNA differences in all cases (Table I). These were used to design sets of PCR primers that allowed the detection of the parent-specific sequences (Figure 1). Initially we obtained a notable background signal using solid phase mini-sequencing, which was not

abolished by washing or minor alterations in the conditions. Subsequent investigation of the cause of the background revealed that short PCR products were present which were not specific to either parent. However, acrylamide gel electrophoresis of the products of the primer extension (Figure 2) revealed that there was a high degree of specificity for the product of appropriate length. All detection primers were tested on mixes of paternal and maternal DNA samples mixed at ratios lower than those expected if paternal mtDNA was present in an embryo (that is, lower than 0.01%) to confirm that we would detect it if present. Using this method we were able to detect less than 0.001% paternal mtDNA after mixing paternal mtDNA into a maternal mtDNA background (Figure 3). No paternal mtDNA was detected in any of the ICSI samples.

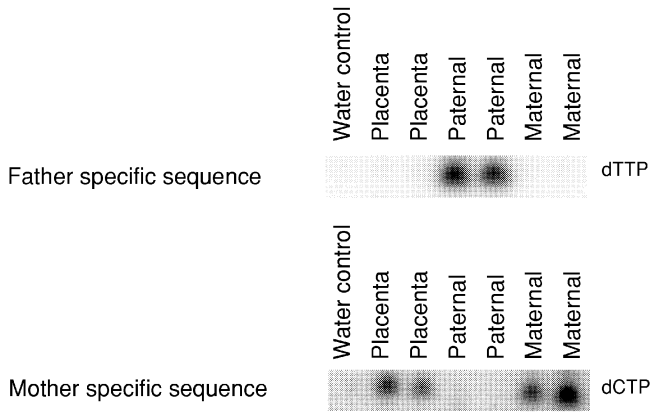

Figure 2. Solid phase mini-sequencing of a family in which there is a polymorphic C->T base change in paternal mtDNA at bp 16269. Radiolabelled primer was detected by phosphorimaging after solid phase mini-sequencing as described in methods. Duplicate samples were analysed in 2 sets of wells. The upper panel shows bands obtained by mini-sequencing using radiolabelled dTTP which corresponds to the paternal sequence. The lower panel shows bands obtained by mini-sequencing using radiolabelled dCTP which corresponds to the maternal sequence. No paternal sequence was detected in the maternal or placental samples. No maternal sequence was detected in the paternal samples. Water controls tested negative for both sequences.

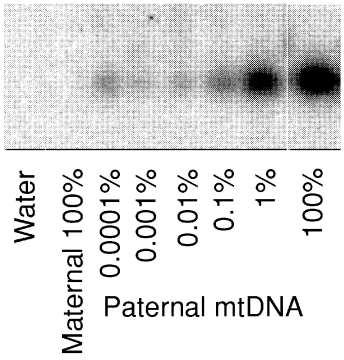

Figure 3. Solid phase mini-sequencing of sequential dilutions of paternal mtDNA (carrying a bp16069T polymorphism) into maternal mtDNA (bp16069C). To determine the sensitivity of the method paternal mtDNA was mixed with maternal mtDNA in ratios encompassing those expected in an embryo in which paternal mtDNA was present. Radiolabelled primer representing paternal sequence was detected by phosphorimaging after solid phase mini-sequencing as described in methods. No paternal sequence was detected in a water negative control or 100% maternal mtDNA but was detectable when paternal mtDNA was mixed with maternal mtDNA down to 0.0001%.

Discussion

In normal individuals there is generally a single population of identical mtDNA (homoplasmy). Two mechanisms probably underlie this. On the maternal side, the so-called mtDNA bottleneck during oogenesis ensures that all of the 100,000 mtDNAs in a normal oocyte arise from a small number or single mtDNA founder. On the paternal side, the mitochondrial bottleneck has an anatomical basis, and it is arguable that the minute contribution of sperm mtDNA to the zygote must be fully functional if it is to win the race against other sperm to the oocyte. While it is commonly held that mtDNA is exclusively maternally inherited, paternal mtDNA can be detected in abnormal human embryos at the blastocyst stage (St John J, 2000) and mouse studies have shown that paternal mitochondria may persist in the zygote for several days (Cummins *et al.,* 1999). Furthermore, ICSI alters both the time course of early development of the zygote (Tesarik *et al.,* 1994) and potentially the dose of the paternal contribution to mtDNA. The use of immature sperm types including epididymal and testicular sperm might increase the risk of paternal mtDNA inheritance. Between meiosis and the end of spermatogenesis there is an

8-10 fold reduction in mtDNA copy number per cell (Hecht *et al.* 1984), reducing the number of paternal mtDNA molecules available for potential transmission in mature spermatocytes. Sperm mitochondria are ubiquinylated during spermatogenesis, this is masked during epididymal passage but unmasked and amplified after fertilisation, targeting the sperm mitochondria for destruction in the 2-4 cell embryo (Sutovsky *et al.*, 2000). This process appears to be dependent on species-specific recognition, raising the possibility that in abnormal sperm development or immature sperm this recognition/destruction system may be defective.

Our adaptation of solid phase minisequencing, has improved its sensitivity and enabled us to exclude levels of down to 0.001% paternal mtDNA in both embryonic and extra embryonic tissues in 11 children born following ICSI using ejaculated sperm, compared with the expected level of 0.1% to 0.5%. It argues against a possible compartmentalization of the injected sperm tail in the fertilized oocyte, which theoretically could lead to relatively high levels of paternal mtDNA in either embryonic or extraembryonic tissues. It lends further support to the results of other groups who have obtained negative results in embryonic tissues, using less sensitive techniques (Danan *et al.,* 1999; Houshmand *et al.,* 1997; Torroni *et al.,* 1998).

Investigating the potential for paternal inheritance of mtDNA is important for 2 reasons. Firstly, ICSI eliminates the selection pressures that normally maintain genetic fitness (Phillipson *et al.,* 2000; Silber *et al.,* 1998). By eliminating the anatomical basis of the mitochondrial bottleneck, ICSI may select for mtDNA mutations causing male infertility. Secondly, population geneticists routinely use mtDNA haplotypes to trace population migrations and infer divergence times based on mtDNA diversity. These studies assume that mtDNA does not recombine. However, recent reanalyses suggest that this critical assumption may be incorrect (Eyre-Walker *et al.,* 1999). The proposal that mtDNA might recombine requires the mixing of mtDNA from 2 disparate lineages. This infers co-existence of paternal and maternal mtDNA in a single cell.

In conclusion, we did not detect paternal mtDNA in blood, buccal swabs, placenta or umbilical cord of children born after ICSI. We have found no evidence that ICSI increases the risk of paternal transmission of mtDNA and hence of mtDNA disorders.

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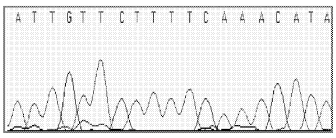
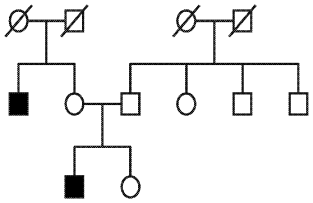
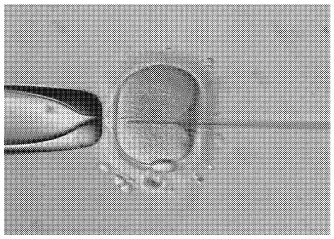
Part III

Counselling and outcome of intracytoplasmic sperm injection







Chapter 7

Reproductive decisions of men with microdeletions of the Y chromosome: the role of genetic counselling

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Abstract

Couples dealing with microdeletions of the Y chromosome have to make decisions about their reproductive future. Do they opt for intracytoplasmic sperm injection (ICSI), artificial insemination with donor insemination (AID) or no treatment? We analysed this decision in 28 couples and investigated the role of the counsellor and the counselling process on the final decision of the couple. Ten counsellors from six fertility clinics in The Netherlands and Belgium were interviewed about their genetic counselling of couples dealing with microdeletions. The answers to the questionnaire were converted to 11 dichotomous variables. Of the 1627 tested men in the six centres, 37 (2.3%) had a microdeletion in the AZFc region, a subregion of the AZF region on the Y chromosome important for normal spermatogenesis. The decisions of 28 of them could be analysed. Most couples chose ICSI (79%). The remaining couples chose donor insemination (7%) or refrained from treatment (14%). Several variables, including the counselling procedure, the counsellor and the available treatments in the fertility centre, influenced the decision of the couple. In conclusion, most couples dealing with microdeletions in the AZF region choose ICSI. Several aspects of the process of genetic counselling appear to be related to the final decision.

Introduction

Men with microdeletions of the Y chromosome and their wives have to make decisions about their reproductive future. Do they choose intracytoplasmic sperm injection (ICSI), artificial insemination with donor semen (AID) or no treatment? Little is known about this decision-making and the role of genetic counselling.

Microdeletions in the azoospermia factor (AZF) region of the Y chromosome have been described in oligozoospermic and azoospermic men (Tiepolo *et al.*, 1976; Ma *et al.,* 1992; Vogt *et al.*, 1992; Reijo *et al.*, 1996; Pryor *et al.*, 1997; Kremer *et al.*, 1998). Most deletions are found in the AZFc region (Simoni *et al.*, 1998). After the introduction of ICSI (Palermo *et al.*, 1992), men with microdeletions in the AZF region could father children despite their severe oligozoospermia or azoospermia. Recently, transmission of microdeletions to sons via ICSI has been described (Silber *et al.*, 1998). So, presumably men with microdeletions will transmit the deletion, as well as the related fertility problem, to their sons.

Many in-vitro fertilization (IVF) clinics offer their patients with severe oligozoospermia or azoospermia testing for microdeletions in the AZF region. If a deletion is found, a decision has to be made by the couple. Different authors have emphasized the importance of genetic counselling in this process (Mau *et al.*, 1997; Pauer *et al.,* 1997; Tuerlings *et al.*, 1997). As stated before, little is known about the impact of having a microdeletion on further decision-making on reproductive options. In the present study, we analysed this decision-making and investigated the relationship between some aspects of the process of genetic counselling and the final decision.

Materials and Methods

In order to obtain information about the genetic counselling and reproductive decisions taken by couples dealing with microdeletions in the AZF region of the Y chromosome, 14 fertility clinics in The Netherlands and Belgium were contacted and asked to participate in the study.

In the participating clinics, counsellors were interviewed about the genetic counselling of couples dealing with microdeletions of the Y chromosome. A questionnaire containing 30 questions was used, focusing on the information given about Y deletions and the consequences for the offspring, the treatment options available and the counsellors' own

opinion about Y deletions. Counselling on ICSI included information about microdeletions on the Y chromosome being the cause of the fertility problem and the risk of transmitting fertility problems to their male offspring. Counselling on AID included the information that there is no increased risk from the use of genetically abnormal spermatozoa. Furthermore, the reproductive decisions of the individual couples were documented. This information was gathered under supervision of a representative of the centre and handled anonymously.

Subsequently, we analysed the relationship between the answers of the counsellor to the questionnaire and the final decision. The answers to the questions were converted to 11 dichotomous variables that were thought to be related to the decisions of the couple (Table I). Statistical analysis was performed by chi-square tests.

Results

Study group

Fourteen IVF centres were contacted to participate in the study (until August 1998). One centre did not respond. In two centres, diagnostic tests to detect Y deletions were not offered to the patients. In two centres, the patients were referred to other centres for diagnostic tests. Three centres offered diagnostic research on Y deletions, but no Y deletions were detected. In the remaining six centres (five in The Netherlands and one in Belgium), 1627 men were tested and 37 men appeared to have a microdeletion in the AZFc region of the Y chromosome (2.3%). One couple did not want to be genetically counselled and eight couples had not taken a decision at the time of the study. These nine couples were excluded from the study.

The mean age of the men in the remaining 28 couples was 34.7 years (range 24–48). The mean age of the women was 31.9 years (range 23–43).

Genetic counselling

The 28 couples in the six centres were counselled by 10 different counsellors: four clinical geneticists, three gynaecologists, two andrologists and one embryologist. The counselling was performed during one to three sessions with one or two counsellors.

Table I. Dichotomous variables related to the counselling of men with Y deletions, questions asked and answers given to the counsellors, and the interpretation of the answers (in italics). Reproductive decisions towards: intracytoplasmic sperm injection (ICSI), artificial insemination with donor semen (AID) or no treatment.

Variables	Questions	Answers
Frequency of counselling about Y deletions	On how many occasions do you counsel couples about Y deletions?	*3 or more: frequent counselling about Y deletions*
Adequacy of counselling about consequences for sons	What do you tell about the consequences of Y deletions for sons?	*Sons inherit the deletion and probably will have fertility problems: adequate counselling about Y deletions*
Thoroughness of counselling about Y deletions	How thoroughly do you counsel couples about Y deletions? (on a scale from 1 to 10)	*6 or more: thorough counselling about Y deletions*
Thoroughness of counselling about ICSI in general	How thoroughly do you counsel couples about ICSI in general? (on a scale from 1 to 10)	*6 or more: thorough counselling about ICSI in general*
Thoroughness of counselling about risks of ICSI for the offspring	How thoroughly do you counsel about risks of ICSI for the offspring? (on a scale from 1 to 10)	*6 or more: thorough counselling about risks of ICSI for the offspring*
Opinion of the counsellor about the seriousness of Y deletions	Do you think that a Y deletion is a serious defect? (on a scale from 1 to 10)	*6 or more: Y deletions are serious defects according to counsellor*
Own decision of the counsellor	If you (or your partner) would have a Y deletion, would you choose ICSI?	*Yes: counsellor chooses for ICSI*
Availability of ICSI in the department of the counsellor	Are both ICSI and AID available in your department?	*No, only ICSI: only ICSI available*
Availability of AID in the department of the counsellor	Are both ICSI and AID available in your department?	*No, only AID: only AID available*
Experience of counsellor	How many men with Y deletions did you counsel?	*6 or more: experienced counsellor*
Directivity of the counsellor	How directive are you as a counsellor? (on a scale from 1 to 10)	*6 or more: directive counsellor*

Couples' reproductive decisions

Twenty-two couples choose ICSI (79%). Of the six couples who did not choose ICSI, two couples opted for AID (7%), and four couples decided to refrain from either form of treatment (14%).

The variables derived from the answers to the questionnaire, and their statistical analysis using chi-square test are shown in Table II. Seven variables were significantly related to the final decision. Four of these seven variables were significantly related to the choice for ICSI: adequate counselling about Y deletions (P = 0.01), thorough counselling about Y deletions (P = 0.002), thorough counselling about risks of ICSI for the offspring (P = 0.02) and only ICSI available in the department of the counsellor (P = 0.04). Three of the seven variables were significantly related to the choice for the alternatives of ICSI: Y deletions are serious defects according to the counsellor (P = 0.02), only AID available in the department of the counsellor (P = 0.002) and directive counsellor (P = 0.002).

Discussion

Our data show that about 80% of the couples dealing with microdeletions in the AZFc region choose ICSI rather than AID, adoption or no treatment. This high frequency is consistent with the findings of a Dutch study describing reproductive decisions of couples dealing with chromosomal abnormalities (Giltay *et al.*, 1998). The decisions of the couples in this study and in our study are not influenced by financial motives, since the ICSI treatment is almost completely reimbursed in the Netherlands and partially in Belgium.

One may conclude that testing for microdeletions is not useful: *with* testing most couples choose ICSI, whereas *without* testing probably most people will also choose ICSI. We do not agree with this conclusion. First of all, a reasonable percentage of the couples decided to refrain from ICSI after testing. Furthermore, little is known about the consequences and risks of ICSI. Therefore we think that we have the obligation to inform the couple about what we do know. We have to inform them in an optimal way and offer all ICSI men with severe oligozoospermia or azoospermia testing for microdeletions in the AZF region. After genetic counselling, the couple can make their own and well-informed choice about their reproduction.

Table II. The variables related to the counselling of 23 men with Y deletions (Table I) and the final decision of the couple

Variables	Frequency of variables (n = 28)	Couples' decision ICSI (n = 22)	Couples' decision No ICSI (n = 6)	$\chi 2$	P-value
Frequent counselling about Y deletions	13	12	1	2.72	0.10
Adequate counselling about Y deletions	13	13	0	6.62	0.01
Thorough counselling about Y deletions	19	18	1	9.17	0.002
Thorough counselling about ICSI in general	16	14	2	1.77	0.18
Thorough counselling about risks of ICSI for the offspring	16	15	1	5.11	0.02
Y deletions are serious defects according to counsellor	8	4	4	5.43	0.02
Counsellor chooses for ICSI	20	17	3	1.72	0.19
only ICSI available	10	10	0	4.24	0.04
only AID available	6	2	4	9.28	0.002
Experienced counsellor	17	15	2	2.40	0.12
Directive counsellor	6	2	4	9.28	0.002

Each counsellor tries to counsel in an objective way and wants to think that the couple makes a free decision. However, this seems not to be the case. Our data show that the decision of the couple to choose ICSI or its alternatives is related to a number of variables of the process of genetic counselling. The clinic of the counsellor is important: if only ICSI is available more couples choose ICSI, and if AID is the only available treatment more couples choose AID. Additionally, the counsellor is important: if he or she has the opinion that microdeletions are serious defects and if he or she counsels in a directive way, more couples refrain from ICSI. Finally, the process of counselling is important: if this process is judged by the counsellor as thorough and adequate, more couples choose ICSI.

We performed a multicentre study to get a large study group. However, the numbers remain too small to perform a multivariate analysis on the data. Because the analysed variables are not independent, care must be taken when interpreting each individual variable. Moreover, we only demonstrated a statistical relationship between the variables and the final decision and not a causal relationship.

Pre-implantation genetic diagnosis (PGD) and sex-selection is an alternative strategy for couples dealing with microdeletions. However, this possibility was not available in the six centres studied. We know that there are clinics in Belgium that offer PGD to couples dealing with microdeletions, but the results have not been published until now.

Only 37 of the 1627 tested men had a microdeletion in the AZFc region (2.3%), which is lower than the frequency in earlier studies (reviewed by Simoni *et al.*, 1998). This may be due to the fact that a majority of the tested men had oligozoospermia and not azoospermia, since ICSI with surgically retrieved spermatozoa is not allowed in The Netherlands. Microdeletions are found more frequently in azoospermic men than in oligozoospermic men (Simoni *et al.*, 1998). An alternative explanation for the relatively low frequency may be publication bias of positive results in earlier studies.

We conclude that most couples dealing with microdeletions of the Y chromosome choose ICSI and that a minority choose an alternative. Several aspects of the process of genetic counselling are related to the decision-making. Each counsellor should be aware of this phenomenon and should try to counsel in an objective and optimal way, so that the couples can make well-informed choices about their reproductive future.

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Chapter 8

Decreased fertilization rate and embryo quality after intracytoplasmic sperm injection in oligozoospermic men with microdeletions in the azoospermia factor c region of the Y chromosome

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Abstract

Microdeletions of the azoospermia factor (AZF) region of the Y chromosome occur in between 1 and 29% of oligozoospermic and azoospermic men, and most deletions are found in the AZFc region. These men can father children when intracytoplasmic sperm injection (ICSI) is used, but the success rate is unclear. Thus, the success rate of 19 ICSI treatments in eight couples with a microdeletion in the AZFc region of the Y chromosome was analysed retrospectively. These were compared with a control group of 239 ICSI treatments in 107 couples undergoing ICSI treatment with ejaculated spermatozoa. The fertilization rate was significantly lower in the group of Y-deleted men (55%; 95% CI: 41–69%) compared with controls (71%; 95% CI: 67–74%; $P < 0.01$). The embryo quality was also significantly poorer among Y-deleted men ($P < 0.001$). Pregnancy, implantation and take-home baby rates were not significantly lower in the Y-deleted group. This study shows that ICSI in oligozoospermic men with microdeletions in the AZFc region of the Y chromosome leads to a lower fertilization rate and poorer embryo quality.

Introduction

The azoospermia factor (AZF) region of the Y chromosome has been described previously (Tiepolo and Zuffardi, 1976), based on the observation of cytogenetic deletions in infertile men. Many studies have investigated the role of this region in spermatogenesis. Until now, it has been suggested that some of the genes in the Yq11 region control spermatogenesis (Ma *et al.*, 1993; Reijo *et al.*, 1995; Lahn and Page, 1997). This region can be divided into three non-overlapping regions of the Y chromosome: AZFa, b and c (Vogt *et al.*, 1996).

Microdeletions of the Y chromosome occur in between 1% and 29% of subfertile men (Foresta *et al.*, 1997; Kremer *et al.*, 1997; Van der Ven *et al.*, 1997). The frequency is dependent on the definition of male subfertility and on the choice of sequence tagged sites used for screening (Simoni *et al.*, 1999). The AZFc locus containing the *DAZ* gene cluster is the most frequently deleted region of the Y chromosome in men with non-obstructive infertility (Kostiner *et al.*, 1998; Simoni *et al.*, 1998). Histologically, these deletions are associated with various spermatogenetic alterations, including Sertoli cell-only syndrome, maturation arrest and hypospermatogenesis.

Following the introduction of intracytoplasmic sperm injection (ICSI), it was possible for men with a microdeletion of the Y chromosome to father children, despite their severe oligozoospermia or azoospermia, using ejaculated or surgically retrieved spermatozoa.

When a microdeletion of the Y chromosome is diagnosed, it is important that a couple is informed about their reproductive options. These options are ICSI, artificial insemination with donor semen, or no treatment. Most infertile couples (79%) with a microdeletion of the Y chromosome choose ICSI as treatment for their fertility problem (Nap *et al.*, 1999). The couple should also take into account the success rates of these options, besides the fact that when ICSI is used, the male offspring inherit the same deletion and presumably the related fertility problem (Mulhall *et al.*, 1997).

Although many reports have detailed the success rate of ICSI treatment, virtually no information is available (other than a few case reports) regarding the success rates of ICSI in couples with severe oligozoospermia due to microdeletions of the Y chromosome. In the present study, the outcome of ICSI treatment using spermatozoa from men with a microdeletion of the Y chromosome was compared with that of ICSI treatment using spermatozoa from oligozoospermic men without this deletion.

Materials and Methods

Patient selection

In our clinic, subfertile men are candidates for ICSI if their ejaculate contains $<10^6$ spermatozoa with propulsive motility (WHO a and b; World Health Organization, 1992). Since 1996, these men have been screened for microdeletions of the AZFa, b and c regions of the Y chromosome (Hoefsloot *et al*., 1997).

The fertilization rate, embryo quality, pregnancy rate, implantation rate and take-home baby rate after ICSI in couples in which the man had microdeletions of the Y chromosome and couples without this genetic disorder were compared retrospectively. Observations were commenced when oocytes were able to be punctured at the time of oocyte retrieval.

The success of ICSI treatment is mainly influenced by maternal age, and whether the subfertility is primary or secondary (Sherins *et al*., 1995; Abdelmassih *et al*., 1996; Stolwijk *et al*., 2000). For this reason, controls with a primary subfertility which had been treated within the same year and at the same age as one of the patients, were considered. Among this group, only couples who had started their first ICSI cycle in 1996 or later were included. Thus, matching was carried out without using a fixed matching ratio.

Because of possible interference with the outcome of ICSI treatment, the following couples were excluded from the control group: (i) chromosomal abnormalities in the male ICSI candidate; (ii) cryopreserved spermatozoa used for ICSI; (iii) using ejaculated spermatozoa from a man with a vaso-vasostomy; and (iv) ICSI treatment performed because of total failure of previous IVF treatment, despite normal sperm parameters.

ICSI treatment

Ovarian stimulation was performed by means of a long protocol of gonadotrophin-releasing hormone (GnRH) agonist, that was started on day 21 of the previous cycle, followed by human menopausal gonadotrophin (HMG). Oocyte retrieval occurred 36 h after HCG injection, and ICSI was performed according to a published technique (Palermo *et al*., 1992). Fertilization rates were scored 18–22 h after the ICSI procedure, with respect to injected oocytes. On day 3 after oocyte retrieval the embryos were scored for cell number and percentage of fragmentation using a modification of a previously published scoring system (Bolton *et al*., 1989): no fragmentation = excellent; <10% fragmentation = good; >10% fragmentation = fair. The best embryos were selected for embryo transfer. Before 1997, a maximum of three embryos was transferred; however, since January 1997

the maximum number of transferred embryos has been two. Clinical pregnancy was defined as a positive (>50 IU/l) urinary ß-HCG test on day 18 after oocyte retrieval. An ongoing pregnancy was defined as one or more gestational sacs *in utero*, 5 weeks after embryo transfer. The implantation rate per embryo was defined as the fraction of transferred embryos developing to gestational sac. The take-home baby rate was defined as a dichotomous variable: one (or more) child per pregnancy was considered to be a positive result, and the ratios were calculated in relation to an embryo transfer and in relation to an oocyte retrieval.

Statistical analysis

Two sample tests according to Student and Wilcoxon have been used with respect to number of oocytes per oocyte retrieval, percentage of injected oocytes per retrieval (injection rate), and the fertilization rate.

Fisher's exact tests were carried out with respect to the categorical variable for embryo quality of the transferred embryos and the dichotomous variables for the occurring of pregnancy, ongoing pregnancy, take-home baby rate per embryo transfer and take-home baby rate per oocyte retrieval. Testing according to Wilcoxon's two sample test was also performed with respect to the implantation rate of embryos transferred. Only SAS procedures were used.

In order to justify the above statistical analyses, a more sophisticated approach was applied by taking into account the patient dependence of cycle results. First, an alternative analysis was performed with respect to fertilization rate on the basis of a general linear mixed model with random patient effects (SAS procedure mixed). In addition, an alternative analysis was carried out with respect to the pregnancy and take-home baby rates on the basis of a logistic regression analysis with random patient effects (SAS macro GLIMMIX). A *P*-value 0.05 was considered to be significant.

Results

Between 1996 and 1998, a total of 300 men with $<10^6$ spermatozoa with propulsive motility in their ejaculate (azoospermia) was screened for microdeletions in the AZF region of the Y chromosome.

Table I. Results ICSI treatment until fertilisation rate of the couples dealing with Y deletion and the controls

	Y deletion 8 couples [a]	Controls 107 couples	P-value
No. of oocytes collection procedures (OPU)	19	239	
No. of cycles with injected oocytes	18	238	
No. of embryo transfers (% of OPU)	17 (89%)	230 (96%)	
Mean ± SD (range) no. of oocytes per OPU	8.1 ± 4.0 (1-20)	9.6 ± 4.9 (1-32)	0.19 [b]
Mean ± SD (range) no. of injected per OPU in %	79 ± 28 (0-100)	82 ± 18 (0-100)	0.39 [b]
Mean ± SE of fertilisation rate in % (95% confidence interval)	55 ± 7 (41,69)	70 ± 2 (67,74)	0.01 [b]
[c] Mean ± SE of fertilisation rate in % (and 95% confidence interval)	54 ± 7 (41,68)	71 ± 2 (68,75)	0.02 [c]

[a] One of these couples had no injected oocytes, because of azoospermia at the time of ICSI
[b] P-values according to Student's two sample test. Because of the skewness of distributions also the nonparametric Wilcoxon's two sample test has been applied, leading to the same conclusion (respectivelyp=0.20, 0.96, 0.03)
[c] Results according to a general linear mixed model with a couple as a random effect

Table II. Embryo quality score of the transferred embryos and their number in each group of the scoring system in couples dealing with Y deletion and the controls

Embryo quality	Definition	Y deletion	Controls
Excellent	No fragmentation	7 (19%)	220 (49%)
Good	<10% fragmentation	10 (27%)	143 (32%)
Fair	Fragmentation >10%	20 (54%)	86 (19%)
Total		**37**	**449**

Significant difference between the two groups with respect to the distribution of embryo quality according to Fisher's exact test ($P < 0.001$)

Table III. Pregnancy, implantation and take home baby rate of the couples dealing with Y deletion and the controls

	Y deletion 17 Embryo Transfers	Controls 230 Embryo Transfers	P-value
Clinical pregnancy rate per embryo transfer (in % ± SE)	3 (18% ± 9%)	73 (32% ± 3%)	0.28 [a]
[b] Clinical pregnancy rate per embryo transfer in % ± SE	18% ± 26%	33% ± 7%	0.25 [b]
Ongoing pregnancy per embryo transfer (in % ± SE)	3 (18% ± 9%)	70 (30% ± 3%)	0.41 [a]
Implantation rate in % ± SE	14% ± 9%	18% ± 2%	0.36 [c]
Take home baby rate per embryo transfer (in % ± SE)	3 (18% ± 9%)	60 [d] (26% ± 3%)	0.57 [a]
[b] Take home baby rate per embryo transfer in % ± SE	17% ± 28%	28% ± 8%	0.42 [b]

[a] P-values according to Fisher's exact test for a 2 × 2 - table
[b] Results according to a generalized linear mixed model (logistic regression analysis with random sample effects)
[c] P-values according to Wilcoxon's two sample test
[d] Including 2 cycles with an ongoing pregnancy, but with unknown outcome at the closing date of data collection

Among these men, eight (2.7%) appeared to have a microdeletion in the AZFc region of the Y chromosome. No microdeletions were detected in the AZFa or b regions. The mean sperm count among these men was $2.3x10^6$ per ml, with 19% propulsive spermatozoa. All eight couples suffered from primary subfertility; the mean (± SD) age was 34 ± 5 years for females, and 36 ± 5 years for males.

The outcome after ICSI treatment until fertilization of couples with an AZFc deletion (n = 8) and control couples (n = 107) is shown in Table I. In the former group, 19 retrieved oocytes resulted in a significantly lower fertilization rate of 55% (95% CI: 41–69%) compared with 71% (95% CI: 67–74%) in the control group (P = 0.01). In addition, the embryo quality was significantly poorer (P < 0.001) among the group of Y-deleted men (Table II).

In 17 of the 19 cycles, embryos could be transferred in the Y-deletion group. No fertilization occurred in two cases: in one case this was because the man had azoospermia at the time of the ICSI procedure; in the other case there was no fertilization of the single oocyte that had been injected. The pregnancy rate, implantation rate and take-home baby rate per embryo transfer were not significantly lower in the Y-deleted group (Table III).

The take-home baby rate was also calculated per oocyte retrieval procedure, but was not significantly different for the Y-deleted and control groups (16% versus 25% respectively). Five healthy female babies were born as a result of the three pregnancies.

Discussion

In this study it was found that ICSI with ejaculated spermatozoa from men with a microdeletion in the AZFc region of the Y chromosome resulted in a reduced fertilization rate and embryo score. Although in IVF the fertilization rate may be affected by sperm and/or oocyte factors, success rates after ICSI are most likely not related to sperm parameters (Mansour *et al.*, 1995; Nagy *et al.*, 1995). The injection rate and number of oocytes were not significantly different between the two groups in this study. Furthermore, it was apparent that the same staff, using the same protocols during the same time period, performed the ICSI procedures.

It remains unknown how spermatozoa with an AZFc deletion influence the fertilization rate and embryo quality. Until now, it has been thought that the main function of the AZFc region of the Y chromosome is its involvement in spermatogenesis. It may be postulated that either the quality of the spermatozoa or the sperm function in fertilization and embryo

development is impaired due to the AZFc deletion. The current study might indicate that Y bearing spermatozoa of these men have a lower chance of fertilization and normal embryo development. If this were the situation, it would only occur in male embryos, and result in a lower male:female ratio. Therefore, it would be interesting to investigate the sex of the supernumerary embryos in those couples with an AZFc deletion.

This hypothesis is supported in the current Y deleted group, because only daughters were born to these couples. However, male offspring have been born after ICSI using spermatozoa from men with an AZFc deletion (Page *et al.*, 1999).

As argued above, it may be important to maximize the available number of good quality embryos in couples with an AZFc deletion, perhaps by attempting to increase the number of oocytes collected. In this way the number of embryos available for transfer can be increased.

No significant difference was found in the take-home baby rate after ICSI treatment in couples with microdeletions in the AZFc region of the Y chromosome, although due to the low frequency of microdeletion of the Y chromosome in oligozoospermic men the number of patients in this study was small. Research on a larger group of men may show an influence on both pregnancy and take-home baby rates. If the pregnancy and take-home baby rates in the current study are considered to be true rates, then increasing the number of ICSI treatments with oocyte retrieval in couples with Y deletions to at least 150 and 375 respectively would be required to reach statistical significance at the 5% level, assuming a power of 80% for the statistical test.

In the present group of patients we were confronted with azoospermia in one patient at the time of ICSI, though pre-treatment semen analysis from this patient revealed oligozoospermia. Men with an AZFc deletion sometimes have azoospermia. In The Netherlands it is not permissible at present to use spermatozoa from epididymal or testicular origin (TESE), and such men can only be treated at a fertility centre in another country. A progressive reduction in sperm number over several months has been described in some men with AZFc deletions (Girardi *et al.*, 1997; Simoni *et al.*, 1997), and it is these men who may benefit from early detection combined with cryopreservation of their semen.

Although in some centres Y chromosome deletion analysis is still not offered, it is feasible that a couple can benefit from testing. First, little is known about the origin of male subfertility and about the consequences and risks of ICSI. Furthermore, it has been shown that a reasonable percentage (21%) of couples with a microdeletion of the Y chromosome

decide to refrain from ICSI treatment (Nap *et al.*, 1999). Therefore, we consider that we have an obligation to inform the couple with regard to their situation, and only then can they make a well-informed choice about their reproductive status. Moreover, as argued above, the finding of a Y chromosome microdeletion might be an adverse prognostic finding for future treatment, and patients must be informed of the situation and possible prognostic aspects (Krausz *et al.*, 2000). Deletions including and extending beyond the AZFc region (AZFb+c and AZFa+b+c) are associated with a total absence of testicular spermatozoa (Silber *et al.*, 1998), and the presence of AZFb deletion is a significantly adverse prognostic finding for TESE (Brandell *et al.*, 1998). Nonetheless, mature spermatozoa have been found in ~50% of azoospermic patients with AZFc deletions. The present study adds to the ongoing discussion that couples with an AZFc deletion show a poorer outcome in ICSI treatment. However, further investigation using larger numbers of couples with AZFc microdeletions of the Y chromosome is required to study the possible prognosis of such deletions on ICSI treatment.

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Chapter 9

A retrospective follow-up study on intracytoplasmic sperm injection

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Abstract

Purpose: Genetic aspects of male subfertility and the novelty of intracytoplasmic sperm injection (ICSI) as a new technique can influence the development of zygotes and children born after ICSI. Therefore, we evaluated the outcome of ICSI compared to in vitro fertilisation.

Methods: Data from medical records of in total 233 pregnancies and the follow-up of 132 children born after IVF and 120 after ICSI were retrospectively analysed.

Results: No differences were found between ICSI and IVF for early embryonic development and obstetric outcome. In both groups the rate of women undergoing prenatal chromosomal diagnosis was low, 30.0%. The congenital malformation rate was 3.0% after IVF and 1.7% after ICSI, which was not significantly different. Follow-up on development of children born after IVF and ICSI also showed no significant differences.

Conclusions: Our results indicate that at this moment ICSI is a safe procedure. However a consistent prospective follow-up is still mandatory to exclude possible risks.

Introduction

It is important to accumulate information about the safety of intracytoplasmic sperm injection (ICSI), because of theoretical risks and the novelty of the procedure. Collecting data on preembryonic development, obstetric and perinatal outcome and follow-up of children born after ICSI could indicate possible deleterious effects of ICSI on the normal development.

Attempts to alleviate male-factor fertility problems through in vitro fertilisation (IVF) have resulted in low rates of success for men with abnormal sperm characteristics. ICSI, a technique that allows microinjection of a single spermatozoon into the oocyte cytoplasm, has been proven a successful alternative in treating male-factor subfertility (Palermo *et al.,* 1992).

Since the introduction of this technique there has been concern about the risks, although theoretically, of the ICSI procedure (De Jonge and Pierce, 1995; Vogt, 1995; Meschede *et al.,* 1995; Patrizio, 1995; Persson *et al.,* 1995): (i) Risks related to the ICSI technique: (a) the manipulative procedure itself, (b) injection of foreign material, (c) mechanical activation of the oocyt, and (d) exclusion of the prezygotic selection processes. (ii) The use of abnormal sperm: (a) an increased frequency of constitutional chromosomal abnormalities (De Braekeleer and Dao, 1991) and/or (b) chromosomal abnormalities in sperm (Moosani *et al.,* 1995), and (c) an underlying 'genetic' disorder (Froster, 1995) which causes male subfertility (e.g. cystic fibrosis and Y chromosome deletions) (Kremer *et al.* , 1997), (d) gamete maturation could be impaired in epididymal or testicular sperm (Ariel *et al.,* 1994).

Micro-injection of spermatozoa, that have been selected from men with combined defects of sperm motility, morphology and concentration might lead to an increased incidence of abnormal embryos. These factors might influence the development of the zygote and increase the risk of abnormal offspring.

There have been only a limited number of studies on the follow-up of ICSI. Most studies describe the successful experiences of ICSI in treating male subfertility (Calderon et al., 1995; Palermo et al., 1995).

At this moment follow-up studies show no abnormalities after ICSI. However Bonduelle *et al.,* (1998a) reports that there is a significant increase in chromosomal abnormalities after ICSI. Reclassification of birth defects of the prospective follow-up study from Brussels showed an increased risk of having a major birth defect (Kurunczuk and Bower, 1997).

Recently Bowen *et al.,* (1998) showed an increased risk of mild delays in mental development at 1 year in children conceived by ICSI. These results are not confirmed by the Brussels group (Bonduelle *et al.,* 1998b).
More follow-up data on ICSI should be evaluated because of theoretical risks, the novelty of the procedure and the limited number of controversial follow-up results. Therefore we evaluated the safety of ICSI by comparing data of ICSI to IVF.

Materials & Methods

Patients included in this study became pregnant following treatment with IVF (group 1) or ICSI (group 2), in the year 1995, at the Reproductive Medicine service of the Institut Dexeus, Barcelona, Spain. In total, 1136 patients started a cycle of IVF or ICSI resulting in 1026 oocyte-collections.
Technical procedures relating to methods of ovarian stimulation, oocyte collection, embryo culture and transfer, sperm preparation and microinsemination procedures were reported earlier (Calderon *et al.,* 1995; Boada *et al.,* 1997). ICSI was performed in cases of male-factor fertility problems with impaired semen parameters or unexplained fertilization failure. Ejaculated semen was used in 90.1% of the cases. 2.7% had a microsurgical epididymal sperm aspiration and 7.2% had a testicular sperm extraction.
Azoospermic men were offered chromosomal analysis, and in cases of congenital absence of the vas deferens, couples were screened for cystic fibrosis mutations. These couples were informed about their possible risks of cystic fibrosis and chromosomal abnormalities and subsequently a prenatal diagnosis was offered.
Couples undergoing IVF or ICSI were also offered the possibility of prenatal chromosomal diagnosis by chorionvillus biopsy or amniocentesis in all cases of ICSI and in advanced maternal age (IVF and ICSI).
We collected data from medical records on (a) early embryonic development, (b) obstetric outcome, (c) perinatal outcome, and (d) follow-up of children born after IVF or ICSI. In all couples included in this study oocytes had been fertilized by either IVF or ICSI. In 11 couples oocytes had been fertilized either by IVF or ICSI. Both treatments were initiated because of mild impaired semen parameters. In this group of patients it was not possible to determine the origin of the developing embryo and they were excluded from the study.

Two hundred thirty-three women had an ongoing pregnancy defined by a positive ultrasound after 8/9 weeks of gestation. Spontaneous abortion (n = 40) or an ectopic pregnancy (n = 3) occurred in 43 of 233 pregnancies. Patients were phoned and asked for cooperation when pregnancies proceeded beyond 20 weeks gestation. They were asked for additional information about obstetric outcome, perinatal outcome and follow-up of the children as noted by their medical doctor. In cases of any abnormality more information was collected from the gynaecologist and/or the pediatrician. With the use of standardized questionnaires we were able to collect complete data on 183 (92%) of 190 pregnancies proceeding beyond 20 weeks gestation.

A widely accepted definition of major malformations was used, namely, malformations that generally cause functional impairment or require surgical correction. The remaining malformations were considered minor (Bonduelle *et al.,* 1996). Weights, lengths and head circumferences at birth and at the time of this study were compared to the Catalan national scale (Cyan *et al.,* 1998). Physical and mental development were also compared to this scale at the age of 1, 3, 5, 7, 9, 12, 15 and 18 months by a paediatrician. Furthermore, additional information on development was evaluated by standardized telephonic questionnaires compared to the age of the children (0.5 untill 1.5 years old).

Data were statistically analysed using Student's *t*-test for comparison between ICSI and IVF. A *P* value of < 0.05 (two-tailed) was taken to represent statitiscal significance.

Results

Overall the mean maternal age was 33.67 years (range 25-44, SD 3.83): for IVF 34.3 years (range 25-44, SD 3.94) and for ICSI, 33.1 years (range 25-44, SD 3.63). The mean maternal age in couples treated with ICSI was significantly ($P < 0.05$) lower than in the IVF-treated couples. The mean paternal age was not different in IVF or ICSI and was overall 35.9 years (range 25-69, SD 5.38).

Preconceptional risk and risks during pregnancy like smoking, medication and diseases during pregnancy were equally divided in both groups.

Early pregnancy data

The fertilisation rate was lower after ICSI (50.6%) than conventional IVF (66.4%), which is probably due to the learning process of ICSI. Embryo quality (Plachot *et al.,* 1990) was the same in IVF and ICSI cycles.

The ongoing pregnancy rate per transfer was 27% after IVF and 23.2% after ICSI. Vanishing twins and triplets defined by the decrease in the number of gestational sacs and/or fetal heart activity per ongoing pregnancy was 9 of 120 (7.5%) in IVF and 7 of 113 (6.2%) in ICSI. The rates of spontaneous abortion before 20 weeks of gestation were 22 of 120 (18.3 %) in IVF and 18 of 113 (15.9 %) in ICSI.

Ectopic pregnancy, an ongoing pregnancy implanted outside the uterus, was observed after IVF in only 3 of 120 (2.5%) pregnancies.

The overall ongoing pregnancy rate after 20 weeks of gestation was 190 of 233 (81.5%).

Chromosomal analysis

One chromosomal abnormality, 45,XY, t(13q,14q) was found in 1 of 23 azoospermic men (4.3%).This couple at risk of inheriting a chromosomal abnormality was informed about possible risks and a prenatal chromosomal diagnosis was offered when a viable pregnancy was established .

Prenatal diagnosis was performed using amniocentesis in all cases. The rate of couples undergoing this diagnosis was 57 of 190 = 30 %, the same rate in both groups. Although this screening was offered in all cases of ICSI, advanced maternal age was the main reason after IVF or ICSI. In two cases of ICSI pregnancies, amniocentesis showed an abnormal karyotype. One was inherited from the father and one was 'de novo'. The rate of a de novo chromosomal abnormality per performed karyotype detected by prenatal diagnosis karyotype is 1/28 (3.6%) after ICSI (Table I).

Table I. Abnormal prenatal karyotypes

Result	Origen	Outcome	Maternal age	Paternal age
45,XY, t(13q,14q)	Inherited father	Healthy liveborn	36	39
45, XO	De novo	Selective abortion *	31	33

* Twin pregnancy, the other fetus had a normal karyotype

Obstetric outcome

The overall mean gestational age was 38 weeks (range 25-42, SD 2.82) and did not differ between the two groups. The rate of multiple pregnancy was the same in IVF and ICSI. Overall 120 of 183 (65.6%) were singletons, 57 of 183 (31.1 %) twins and 6 of 183 (3.3%) triplets.

Prematurity, live birth before 37 weeks of gestation, occurred in 22 of 96 (22.9%) after IVF and in 15 of 87 (17.2%) after ICSI. Prematurity was associated mainly with multiple pregnancy, and subsequently there was an increase in problems occurring at birth, 16% in twins and 43% in triplets. Problems occurring at birth were the same in the groups of children born after IVF, 12 of 132 (9.1%), or ICSI, 11 of 120 (8.3%). All these children needed to spend some time in the incubator for observation due to prematurity. Additional problems at birth were of respiratory (n = 5), cerebral (n = 2) and intestinal (n = 1) origin.

The mode of delivery is summarized in Table II. The rate of deliveries without any problems (spontaneous vaginal and elective cesarean deliveries) was 66.7%. The remaining cases had instrumental vaginal or urgent cesarean deliveries. No statistical differences were discovered between the two groups.

Table II. Mode of delivery

Mode of delivery	IVF (n = 96)	ICSI (n = 87)	TOTAL (n = 183)
Spontaneous vaginal delivery	37/96 (38.5%)	27/87 (31.0%)	64/183 (34.9%)
Instrumental vaginal delivery	6/96 (6.3%)	8/87 (9.2%)	14/183 (7.7%)
Elective cesarean section	28/96 (29.2%)	30/87 (34.5%)	58/183 (31.7%)
Cesarean section on indication	25/96 (26.0%)	22/87 (25.3%)	47/183 (25.7%)

Perinatal and neonatal data

The number of children born from 183 pregnancies beyond 20 weeks gestation was 252 (132 IVF and 120 ICSI).

Stillbirth, intrauterine fetal death after 20 weeks of gestation, was 4 of 132 (3.0%) and 1 of 120 (0.8%) after IVF and ICSI respectively. The total of liveborns was 247: 128 after IVF and 119 after ICSI.

The early neonatal death, death of a live-born infant during the first days after birth, was 1 of 128 = 0.8% in IVF and 2 of 117 = 1.7% in ICSI. Tables III and IV show the perinatal mortality cases, the sum of stillbirth and early neonatal death (no statistical differences).

Table III. Perinatal mortality

Definition	IVF	ICSI	Total
Stillbirth	4/132 (3.0%)	1/120 (0.8%)	5/252 (2.0%)
Early neonatal death	1/128 (0.8%)	2/117 (1.7%)	3/245 (1.2%)
Perinatal mortality	3.8%	2.5%	3.2%

Table IV. Clinical history of perinatal mortality

Number of children	Gestational age	IVF / ICSI	Cause
1	34.5	IVF	Intrauterine growth retardation in twin pregnancy
2	25	IVF	Cervical incompetence
1	41	IVF	Fetal Asphexia (occlussion of birth cord)
1	37.5	IVF	Exencefaly (died 3 days after birth)
1	28.5	ICSI	Abruptio placenta
2	26	ICSI	Cerebral hemorrhage died 2 / 4 days after birth *

* In this triple pregnancy the third child died because of hydrocephalus after 4 months of age

The female / male ratio was equally divided and there were no differences between children born after IVF and ICSI.

Neonatal measurements for 252 children are listed in Table V. Birthweight and head circumference compared to the gestational age were not significantly different between both groups. Children born after ICSI were significantly 1.1 cm less tall than children born after IVF. However this difference was within the normal range of length compared to gestational age in the national catalan register.

Table V. Neonatal measurements

Method	Birthweight (g)	Birthlength (cm)	Head circumference (cm)
IVF (n = 132)	2939	48.9 *	34.6
SD	695.7	2.7	1.9
ICSI (n = 120)	2857	47.8 *	33.8
SD	575.3	3.6	2.0
Total (n = 252)	2898	48.4	34.2
SD	SD= 654.7	SD = 3.3	SD = 2.0

* P-value = 0.025 in t-test

Congenital malformations are listed in Table VI. The rate of major congenital malformation was 4 of 132 (3.0%) after IVF and 2 of 120 (1.7%) after ICSI, the differences being nonsignificant. Minor malformations were not observed in medical reports or in telephonic standardized questionairres.

Table VI. Congenital malformations

	Malformation	
IVF	**ICSI**	**Total**
4/132 (3.0%)	2/120 (1.7%)	6/252 (2.4%)
Exencefaly	Pes equinovarus	
Hemivertebrae	Hydronephrosis*	
Hydronefrosis *		
Vesico-ureteric reflux *		

* Clinical evaluation showed no gross anatomical genito-urinary tract malformation

Follow-up of children born after IVF and ICSI

All children surviving the perinatal period (n = 244) were between 0.5 and 1.5 years old at the time of the study. Follow-up data were compared to the Catalan national developmental scale. Measurements of weights, lengths and head circumferences of the children at the time of the study were not significantly different between IVF and ICSI.

Problems occurring during the development of children born after IVF and ICSI are divided into physical problems or mental developmental problems. We found no significant differences between IVF and ICSI for physical and/or mental development. Developmental problems, both physical and mental, were discovered in 3 of 127 (2.4%) children after IVF and in 4 of 117 (3.4%) after ICSI. The developmental delays were minor and of unknown origin.

There were two children who needed medical treatment: one child born after IVF had recurrent convulsions and mental developmental delay and one child born after ICSI needed treatment because of hypothyroidism.

Discussion

It is important to accumulate information on ICSI, whether the zygotes develop normally into children who do not show more abnormalities at birth and during their development. This is important not only because of the risks on theoretical grounds, but also because of the novelty of this technique.

The effects of micromanipulation during ICSI are probably limited. Our results did not show any difference on the early embryonic development between the two groups. This has also been shown in animal models (Bras *et al.,* 1994; Motoisho *et al.,* 1996). This study and others (Calderon *et al.,* 1995; Wisanto *et al.,* 1996) indicate a limited effect on early embryonic development. However, deleterious effects could still be discovered and there is a risk of parthenogenetically activating an oocyte. This could induce a higher incidence of maternally derived chromosomal abnormalities, especially in women of advanced age.

We did not discover any significant differences between IVF and ICSI for obstetric outcome, perinatal outcome and follow-up of children born after ICSI. The congenital malformation rate after ICSI was not significantly different from IVF and seemed to be in the same range of the national register 1.6 % (Salvador *et al.,* 1997).

The relatively high rate of cesarean section, prematurity, and problems at birth in our study could be caused by increased maternal age and/or multiple pregnancy due to multiple embryo transfer. The high rate of cesarean section after IVF or ICSI might also be due to the final decision of the physician and couple, choosing, in their opinion, the best method of delivery after long-term fertility problems.

At this moment follow-up studies (Bonduelle *et al.,* 1996; Bonduelle *et al.* 1998a/b; Govaerts *et al.* 1998; Palermo *et al.* 1996; van Steirteghem *et al.,* 1998; Wennerholm *et al.* 1996) show a normal obstetric outcome, no increase in congenital malformations and normal development of children born after ICSI. However, there are some concerns that ICSI might somehow be responsible for an increased frequency of chromosomal abnormalities and fertility problems in children born after ICSI (Bonduelle *et al.,* 1998a; In 't Veld *et al.,* 1995; Kremer *et al.,* 1997). At present they do not show phenotypic abnormalities in children born after ICSI (Bonduelle *et al.,* 1998b). Moreover, Bowen *et al.,* (1998) showed a significant developmental delay in children born after ICSI. Our results show a normal development of children born after ICSI, but the national Catalan developmental scale (Cyan *et al.,* 1998) may not be accurate enough to detect developmental impairment after ICSI.

Prospective studies are prone to detect a higher rate of abnormalities than retrospective studies (Simpson, 1996). We did not discover any minor malformation, which could be due to our retrospective study design. Comparing data on an international basis can be influenced by the difference in data collection. Most centres have only a limited and retrospective study on the risks in ICSI (van Steirteghem *et al.,* 1998). Conclusions on the risks in ICSI can thus be controversial (Bonduelle *et al.,* 1996; Bonduelle *et al.,* 1998a/b; Bowen *et al.,* 1998; Van Steirteghem *et al.,* 1998).

Prenatal chromosomal diagnosis in our centre in the year 1995 was 30% in both groups, although couples were informed about the genetic risks of ICSI. A low uptake of prenatal diagnosis in ICSI is reported earlier (Payne and Matthews, 1995). Because of the risk of miscarriage, the present follow-up results, and the unknown phenotypic consequence of some chromosomal abnormalities, people may decide not to have a prenatal chromosomal diagnosis. In the prospective study from Brussels (Bonduelle *et al.,* 1998a) the uptake of prenatal diagnosis was 55%.

We discovered one case of a de novo chromosomal abnormality (1/28; 3.6%). The numbers are too small to be of statistical value. Small numbers and screening patients at

high risk could be responsible for high frequency of chromosomal abnormalities (In 't Veld *et al.,* 1995).

Male candidates for ICSI should have a detailed genetic work-up: family history, chromosomal analysis, detection genes involved in spermatogenesis, and genetic counseling (Tuerlings *et al.,* 1997). It is important to indicate the group at risk for 'genetic' abnormalities, although their origin is difficult to detect (Bonduelle *et al.,* 1998a; Van Opstal *et al.,* 1997). The affected couple needs to understand the risks for the offspring and what preventive measures can be taken. In some cases of genetic abnormalities the couple can be offered a prenatal diagnosis (Baschat *et al.,* 1996; Testart *et al.,* 1996).

More investigation is needed concerning genetic causes in males selected for ICSI and the risk(s) for the offspring. Our results show that ICSI is a safe procedure, but international prospective follow-up is still mandatory.

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General Discussion

General Discussion

This thesis describes the familial occurrence and the search for genes involved in male subfertility together with its clinical consequences. In **Part I** we presented the results of the family studies in male subfertility and the familial occurrence and phenotypes of subfertile men. In **Part 2** the search for different genes involved in human male subfertility is described. No new genes could be identified using linkage analysis and candidate gene research. In the last part, **3**, of this thesis we presented our studies on the clinical implications of the genetic aspects of male subfertility. The influence of counselling, the decision-making and the outcome of treating couples dealing with microdeletions of the Y chromosome is described. Lastly, we presented a retrospective follow-up study on ICSI. Up till now ICSI seems to be a safe procedure, but prospective follow-up studies are still needed to warrant the safety of ICSI.

Family studies in male subfertility

We showed that male subfertility appears to have a familial occurrence. Autosomal recessive, sex limited autosomal dominant or X-linked inheritance may be involved. However, fertility problems among relatives are underestimated using a family history, probably due to information bias. Knowledge of fertility problems travels selectively among families causing substantial misclassification. This misclassification hinders quantitative assessment of familial clustering.

Identification of subfertile men with a positive family history may be influenced by this "*taboo bias*". Male subfertility is a difficult topic to discuss. When taking a family history we obtain data on fertility problems in the family second handed. Involuntary childlessness may be the only hint that the couple faced fertility problems. The exact origin of the fertility problem among male relatives can only be identified by performing the standard clinical investigation.

The taboo on fertility problems among subfertile men and their family members has hindered the coopertion undergoing a clinical investigation on fertility problems in these relatives.

On the other side the hypothesis of a familial occurrence of male subfertility may also be discussed because of another bias called: recall bias. In other words differences found in

this case control study may be caused by the fact that subfertile men are better informed on fertility problems in their families than their controls. Our study confirms the earlier results on the familial occurrence of male subfertility (Budde *et al.,* 1984; Lilford *et al.,* 1994; Meschede *et al.,* 2000). A new finding in this family study is the increase of male subfertility among men on the maternal side of the subfertile men. Therefore we hypothesize an X-linked inheritance in these families. A recent study on the abundance of X-linked expressed in mice spermatogonia may support this hypothesis (Wang *et al.,* 2001).

In the study on the phenotypic characteristics it is shown that subfertile men with a familial occurrence of male subfertility more often have normal levels of FSH and LH. This phenomenon is also observed in men with microdeletions in the AZFc regions. The pathogenesis of the genetic aspects in these men probably disturbs spermatogenesis without interfering with the hormonal function of the Sertoli cells. It should also be noted that studying the single cases of male subfertility show higher levels of FSH sometimes accompanied by cryptorchidism. These observations are stimulated by the fact that some candidate genes of cryptorchidism have been described (Krausz *et al.,* 2000; Gianotten *et al.,* 2001).

This clinically well-defined group of subfertile men was the basis for our search on the molecular genetic aetiology of male subfertile.

Molecular genetic studies in male subfertility

We searched for the genetic aetiology of male subfertility using two strategies: (a) Linkage analysis and (b) Candidate gene analysis. Unfortunately, we have not been able to identify new genes involved in male subfertility.

Two out of 253 families were cooperative and suitable for a linkage analysis. In this thesis we described X-linkage analysis in one family with 5 subfertile maternal nephews. The pedigree suggested an X-linked inheritance, but this could not confirm by molecular linkage analysis. So we hypothesize the existence of an autosomal dominant trait of male subfertility with sex limited expression.

In addition we investigated the role of the candidate genes DAZLA and the Androgen receptor gene in male subfertility. In the DAZLA gene no pathogenic mutations could be

identified. In addition we found no correlation between the length of the CAG repeat in the AR gene and male subfertility.

Two factors may be important in the difficulty to identify the molecular genetic origin of male subfertility. The frequency of chromosomal abnormalities and microdeletions of the Y chromosome is higher in azoospermic men than in men with an OAT. Our group of subfertile men mainly have an OAT instead of an azoospermia. The size of the group subfertile men at risk for a genetic aetiology like azoospermic men may be too small. Secondly, candidate genes in male subfertility like DAZLA are first identified in the mouse model. Candidate genes, although proven in knock out animal models, may not always be important in humans.

ICSI is the choice of treatment in severe male subfertility nowadays and bypasses a number of stages of natural fertilization. There is a risk that ICSI may increase the transmission of paternal mitochondrial DNA. Knowledge of the fate and transmission of paternal mitochondrial DNA is important since mutations in mitochondrial DNA have been described in oligozoospermic males. This risk seems limited, as we found no evidence that ICSI increases the risk of paternal transmission.

Counselling and outcome of intracytoplasmic sperm injection

Counselling the couple on the genetic aspects of male subfertility enables them to make their own and well-informed choice about their reproduction. Therefore it is important to study the clinical implications of the genetic aspects in male subfertility. These aspects influence the counselling, decision-making and outcome of the treatment. It is shown that most couples dealing with a microdeletion of the Y chromosome choose ICSI. Counsellors should be aware of the phenomenon that several aspects of genetic counselling are related to the decision making of the couple as shown in couples dealing with microdeletions of the Y chromosome. Probably this is also the case when counselling couples with other forms of male subfertility with a genetic origin. In this thesis it is shown that most couples dealing with a microdeletion of the Y chromosome choose ICSI. In a study by Giltay *et al.,* (1999), the majority (56%) of the couples with a chromosome abnormality present in the subfertile male partner did not refrain from ICSI.

Problems in studying counselling and reproductive decisions in genetically determined male subfertility are the relatively low frequency of each specific genetic abnormality

among subfertile men. Another important aspect is that it seems unethical to perform a study in which some couples get counselling and others not. However, this theoretical study design is necessary to evaluate correctly the influence of the diverse aspects of counselling and decision-making.

When a microdeletion of the Y chromosome is diagnosed our study shows that most couples choose ICSI (Nap *et al.,* 1999). The couple should also take into account that the success rates may be decreased when using ICSI, besides the fact that the male offspring inherits the same deletion and presumably the related fertility problem (Mulhall *et al.,* 1997). We demonstrated a decreased fertilisation rate and embryo quality after ICSI in oligozoospermic men with microdeletions in the AZF c region of the Y chromosome. The pregnancy rate and take home baby rate is also lower among these couples, but larger numbers of couples are required to study the possible prognosis of such deletions on ICSI treatment.

In general, it is of outmost importance to investigate the safety of ICSI as a new assisted reproductive technique. Therefore, in every clinic performing ICSI follow-up studies are necessary. In a retrospective follow-up study we have shown that ICSI seems to be a safe procedure. However the retrospective study design, the small number of pregnancies after ICSI with surgical retrieved spermatozoa and the inability to study the long term follow-up, influences the accuracy of this and other studies.

Future follow-up study by Bonduelle *et al.,* (2001) confirms earlier results and shows no increase of congenital malformations even after ICSI in men with very low sperm parameters. Althlough, others find that the number of de novo chromosomal abnormalities in ICSI children is increased (1.6%) especially after the use of testicular sperm (Bajirova *et al.,* 2001). Investigating the safety of ICSI needs a blinded study design with appropriate control groups and data collection that allows correction of confounding factors.

Guidelines for the counselling of couples undergoing ICSI

How should we apply the current knowledge on the genetic aspects of male subfertility and the outcome of ICSI in clinical practice? The couple should be informed in an optimal and objective way so they can make their own well-informed decision, desirably based on (inter)national guidelines. A questionnaire from the European Society of Human

Reproduction (ESHRE) on the way of screening and counselling of ICSI patients in the different countries shows that there are many differences (Kremer *et al.,* 2000).

First the couple should be informed that the aetiology of male subfertility is often unknown and subsequently no other treatments are available. Most of the time ICSI remains the only treatment for the couple to have offspring of both partners. But people should also have the opportunity to choose for donor insemination, adoption or to remain childless.

The information on the complications of the medication and the chance of multiple pregnancies in ICSI should be similar as in conventional IVF. Furthermore the information should be focussed on the specific theoretical risks of ICSI. These can be divided into the theoretical risks of the ICSI procedure itself or the risks of using abnormal sperm.

The risk of using abnormal sperm is important, because of the increased frequency of a chromosomal abnormality in subfertile men and the possibility of a microdeletion of the Y chromosome. The investigation of these two possible genetic abnormalities should be offered to the subfertile men of the couple undergoing ICSI. In cases of a genetic abnormality a (genetic) counsellor can inform them on the aetiology and the consequences. In cases of a CBAVD a CFTR mutation screening should be offered to the male and female partner following the same procedure as mentioned above.

At this moment it is known that there is an increase of male subfertility among relatives of subfertile men. Genetic factors probably play an important role, but couples can not be informed on the exact risk of transmitting the fertility problem to the offspring. Moreover, a genetic factor cannot be ruled out even if the family history does not show a (male) relative with fertility problems. The value of family history in male subfertility should still be evaluated. In some particular cases it may be helpful finding the correct diagnosis and counsel the couple, like in Kartagener's syndrome and globozoospermia.

At this moment there is no evidence that ultrasound during a pregancy established after ICSI is indicated. Invasive prenatal karyotyping after ICSI is discussed, because of the increased risk of de novo chromosomal abnormality in children born after ICSI (1.66% vs. 0.8%). However the risk is only slightly increased and the procedure can induce a miscarriage. Moreover the chromosomal abnormalities are often sex chromosomal abnormalities and balanced translocations and the risks for the offspring seem limited. Care must be taken that many ICSI couples choose advanced ultrasound exam instead of screening the ICSI offspring. This is still under debate and more research is needed to indicate that advanced ultrasound is an appropriate screening device in ICSI pregnancies.

Overall, couples should be informed that ICSI seems to be a safe and successful treatment. Unfortunately, the long-term follow-up data on ICSI are unknown and the couples and the professional people involved in the treatment of ICSI have to deal with these uncertainties. In the Netherlands the Dutch Society of Obstetrics and Gynaecology have provided a patient information brochure, documenting information and bottlenecks when choosing ICSI (Giltay *et al.,* 2001).

Research on male subfertility

In the last decades no new treatment in male subfertility has been successful. Only in the rare case of a deficiency of gonadotropic hormones subfertile men can be treated with pulsatile Gonadotropin Releasing Hormones (GnRH) or gonadotropins (Nieschlag, 2000). Other treatments do not focus on the improvement of semen parameters and this is stimulated by the fact that the exact aetiology of male subfertility is unknown.

Evidence based medicine is considered as the state of the art in good clinical practice. However, medical doctors and patients often seek treatments that are based on theoretical ground or are so called experienced or authority based (Comhaire, 2000). This tendency is stimulated by the fact that often no cause is found and no treatment is available. In this time era the dominating factor in management decisions should not be based on the physician's personal authority and experience. The situation makes it tempting for some physicians to apply innumerable empirical therapeutic procedures whose effectiveness remains uncertain. Many errors in judgement - not only in andrology – can be attributed to this attraction to meddlesome but unproven treatments.

Further research should be performed by the rules of evidence-based andrology. Hopefully, because of the decreasing taboo on sexuality and probably on fertility problems it will become easier to study male subfertility. Research depends on the cooperation of subfertile men.

In our studies we were confronted with the taboo on discussing fertility problems. The impact of fertility problems on sociopsychological factors influences coping with fertility problems and the supposed stigma associated with subfertility. For instance in research on donor insemination it is shown that the stigma associated with infertility forces secrecy and lack of openness (Turner and Coyle, 2000). In a recent study by Conrad *et al.,* (2001)

it was shown that some subfertile men developed psychosomatic problems coping with the fertility problems, so called alexithymia.
The last decades it is commonly known that fertility problems can be caused by female as well as male factors. This development changed the position of men in their contribution to reproduction. During history and in different cultures men were considered the head of a family associated with strength and leadership. Subfertility seems to be controversial with these aspects. One may now consider a new explanation for the story in the old-testament of Abraham and Sarah who remained childless. The current explanation is that Sarah suffered from fertility problems. It is time to consider the fact that Abraham successfully treated his fertility problem marrying a young superfertile woman.
The changes in the western world are gradually influencing the position of the modern man and will eventually influence his social position in reproduction. Hopefully research on male subfertility will benefit from these changes.

Concluding remarks

In this thesis we have found clinical evidence for still unknown genetic factors in male subfertility. This knowledge can be used for counselling the couple dealing with male subfertility. It is important to continue the data collection on clinical characteristics and family history of subfertile men. A large number of clinically well-defined male subfertility may facilitate the search for new candidate genes involved in male subfertility. Male subfertility represents a heterogeneous etiological entity. There is a need for more basic research into the causes and the clinical approach of male subfertility.
The clinical impact of these genetic factors involved in male subfertility deserves further study. The dialogue between people working in the field of reproductive medicine and molecular geneticists will be required to imply new genetic knowledge in clinical practice.
Important aspects for future research are cost-effectiveness of genetic aspects and diverse ethical consequences. It can be argued if people benefit from information on the (genetic) risks in ICSI and the change of transmitting fertility problems. Moreover, people have the right choosing not to be informed on the genetic aspects of male subfertility. The increasing knowledge on the genetic origin of disorders, like male subfertility, will probably lead to more ethical concerns.

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Afwegingen bij de keuze voor ICSI

NVOG Patiëntenvoorlichting

Afwegingen bij de keuze voor ICSI

Folders en brochures van de NVOG behandelen verschillende verloskundige en gynaecologische klachten, aandoeningen, onderzoeken en behandelingen. Zo krijgt u een beeld van wat u normaliter aan zorg en voorlichting kunt verwachten. Soms geeft de arts u andere informatie of adviezen, bijvoorbeeld omdat uw situatie anders is of omdat men in het ziekenhuis andere procedures volgt.
Schriftelijke voorlichting is altijd een aanvulling op het gesprek met de arts. Daarom zijn de NVOG en de NVU juridisch niet aansprakelijk voor eventuele tekortkomingen van deze folder. Wel hebben de Commissies Patiëntenvoorlichting van de NVOG en de NVU zeer veel aandacht besteed aan de inhoud. Dit betekent dat er geen belangrijke fouten in deze folder staan, en dat de meerderheid van de Nederlandse gynaecologen en urologen het eens is met de inhoud. Het concept is inhoudelijk goedgekeurd door de Werkgroep Reproductieve Genetica.
Wij hopen dat u met deze informatie weloverwogen beslissingen kunt nemen. Andere folders en brochures op het gebied van de verloskunde, gynaecologie en voortplantingsgeneeskunde kunt u vinden op de website van de NVOG: http://www.nvog.nl , rubriek voorlichting.

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INHOUDSOPGAVE

1 INLEIDING

ICSI is de afkorting van intracytoplasmatische sperma-injectie. Men brengt hierbij in het laboratorium één zaadcel via een zeer dunne naald in één eicel om deze te bevruchten. Het is dus een behandeling om een zwangerschap te laten ontstaan.
In deze folder gaan we kort in op de redenen voor een ICSI- behandeling en het verloop ervan. De gynaecoloog en de patiëntenvereniging Freya kunnen u meer informatie geven. Ook is een cd-rom verkrijgbaar die de behandeling in beeld en geluid uitlegt.
Hier komen vooral gezondheidsrisico's aan bod. Het accent ligt op de gezondheidsrisico's voor het kind en de mogelijke onderzoeken voor het begin van de behandeling en tijdens de zwangerschap.

2 WAT ZIJN REDENEN VOOR ICSI?

De belangrijkste reden voor een ICSI-behandeling is een zeer laag aantal beweeglijke zaadcellen. Ook als na twee behandelingen met IVF (in vitro fertilisatie) geen bevruchting is opgetreden of als zich antistoffen in het sperma bevinden, kan de mogelijkheid van ICSI ter sprake komen.

3 HOE GEBEURT ICSI?

Bij een ICSI-behandeling dient men net als bij IVF hormonen aan de vrouw toe. Deze hormonen laten in de eierstokken een groot aantal follikels (met vocht gevulde blaasjes) groeien. In iedere follikel bevindt zich een eicel. Na ongeveer twee weken hormoonbehandeling prikt de arts deze follikels onder echo-controle aan. De zo verkregen eicellen bevrucht men daarna in het laboratorium. Als er uit de bevruchte eicellen embryo's ontstaan, plaatst de arts die een paar dagen later in de baarmoeder.
Het verschil tussen IVF en ICSI is de manier waarop de bevruchting in het laboratorium gebeurt. Bij IVF brengt men één eicel samen met ongeveer 100.000 zaadcellen en wacht

men op spontane bevruchting door een van de zaadcellen. Bij ICSI brengt een laboratoriummedewerker één enkele zaadcel in één eicel.

4 GEZONDHEIDSRISICO'S VOOR DE VROUW BIJ ICSI

Zowel bij ICSI als bij IVF worden de eierstokken met hormonen gestimuleerd en aangeprikt, en plaatst de arts meestal twee of soms meer embryo's in de baarmoeder. De zeldzame complicaties die hierbij kunnen ontstaan, zijn voor beide behandelingen dezelfde:

- overstimulatie, een ernstig ziektebeeld met klachten van snelle gewichtstoename, hevige buikpijn en/of misselijkheid,
- bloeding of infectie na het aanprikken van follikels.

De gezondheidsrisico's van deze behandelingen op langere termijn voor vrouwen zijn onbekend. Zowel bij IVF als bij ICSI geeft men hormonen om follikels te laten groeien. Daardoor maken de eierstokken veel oestrogene hormonen. Er is weliswaar een verband aangetoond tussen langdurig gebruik van oestrogenen voor andere doeleinden en een licht verhoogde kans op borst- en baarmoederkanker, maar een dergelijke relatie is tot nu na IVF- en ICSI-behandelingen niet gevonden. Gegevens over deze risico's op de lange termijn zijn echter nog niet beschikbaar. Hetzelfde geldt voor de kans op eierstokkanker. Als gevolg van beschadiging door het rijpen en aanprikken van meerdere follikels is theoretisch een verhoogde kans op dit soort kanker denkbaar.

Ook hier geldt: tot nu toe is geen verhoogde kans op eierstokkanker aangetoond, maar gegevens over de gezondheid van vrouwen vele jaren na een IVF- of ICSI-behandeling zijn er nog niet.

5 GEZONDHEIDSRISICO'S VOOR KINDEREN DIE NA ICSI GEBOREN ZIJN

5.1 Risico's die samenhangen met de bevruchting buiten het lichaam

Zowel bij IVF als bij ICSI vindt bevruchting buiten het lichaam plaats. Dit brengt voor beide behandelingen (theoretische) risico's mee, die bij ICSI niet minder zijn dan bij IVF. Zij hangen samen met (onbekende) effecten van de gebruikte hormoonbehandelingen om veel follikels te laten rijpen en om in de baarmoeder geplaatste embryo's zo goed mogelijk te laten innestelen en groeien.

Daarnaast zijn er de risico's die samenhangen met het verblijf van de te bevruchten eicel buiten het lichaam. In het verleden zijn in Nederland publicaties verschenen over o.a. hepatitis (geelzucht)-infecties en bevruchting door een zaadcel niet van de partner. Door middel van zeer strenge veiligheidsvoorschriften die men aan het laboratorium stelt, probeert men dit soort risico's zo veel mogelijk te voorkomen.

5.2 Risico's die samenhangen met het terugplaatsen van meer embryo's

Als de arts meer embryo's terugplaatst, is niet alleen de kans op een zwangerschap groter, maar ook de kans op een meerling. Ouders die sterk naar een kind verlangen, verwelkomen meerlingen vaak met open armen. Meestal realiseren zij zich niet dat een meerlingzwangerschap nogal eens problemen voor de kinderen met zich meebrengt. Zo komt vroeggeboorte bij tweelingen viermaal vaker voor dan bij eenlingen, en is de kans op sterfte tienmaal groter. Daarnaast hebben kinderen die te vroeg geboren worden ook meer kans op een vertraagde ontwikkeling.

5.3 Risico's die samenhangen met de ICSI-procedure

Bij ICSI brengt men één zaadcel met een injectienaald in een eicel. Men beschadigt zo de wand van de eicel, en bovendien komt de punt van de injectienaald binnenin de eicel. Ook de vloeistof waarin de zaadcel in het laboratorium wordt bewaard, komt in de eicel terecht. Of dit alles voor het kind op lange termijn gevolgen heeft, is nog niet bekend.

Bij een spontane bevruchting (ook bij IVF) proberen vele zaadcellen tegelijk in de eicel door te dringen.

Uiteindelijk lukt het slechts één zaadcel om de eicel te bevruchten. Men spreekt daarom wel van natuurlijke selectie (een wedstrijd tussen zaadcellen waarbij er maar één winnaar is). Bij ICSI kiest men altijd een normaal uitziende zaadcel, maar of dit 'de beste' is, valt niet te zeggen. De natuurlijke selectie ontbreekt dus.

5.4 Risico's die samenhangen met de reden voor ICSI

Mannelijke vruchtbaarheidsproblemen zijn de belangrijkste redenen voor ICSI. Soms speelt bij deze problemen erfelijkheid een rol.

Chromosoomafwijkingen bij de man

Chromosomen dragen onze erfelijke informatie. Ze bevinden zich in de celkernen van alle cellen. Elke celkern bevat 23 paar chromosomen, waarvan twee geslachtschromosomen.

Geslachtschromosomen geeft men aan met de letters X en Y. Zo hebben vrouwen twee X-chromosomen (XX), en mannen een X- en een Y-chromosoom (XY).
Bij mannen met vruchtbaarheidsproblemen komen twee soorten chromosoomafwijkingen voor: afwijkingen van de geslachtschromosomen en afwijkingen van de autosomen (alle andere chromosomen). Bij mannen die verminderd vruchtbaar zijn, komt ongeveer vijfmaal vaker een chromosoomafwijking voor dan in de algemene bevolking.
Bij een afwijking van de *geslachtschromosomen* heeft een man bijvoorbeeld een extra geslachtschromosoom X of Y. Mannen met zo'n chromosoomafwijking hebben behalve de vruchtbaarheidsproblemen vaak geen duidelijke andere verschijnselen.
Bij een afwijking van de *autosomen* bestaat vaak de situatie dat twee stukken van twee verschillende chromosomen onderling van plaats verwisseld zijn (gebalanceerde translocatie). Bij deze man of vrouw zijn er verder geen verschijnselen. Iemand met een gebalanceerde translocatie heeft een grotere kans op het krijgen van een kind met een ongebalanceerde chromosoomafwijking, waarbij bijvoorbeeld een stukje chromosoom ontbreekt.
Kinderen met zo'n ongebalanceerde chromosoomafwijking zijn altijd verstandelijk gehandicapt en hebben ook vaak ernstige aangeboren afwijkingen. Soms zijn de afwijkingen zo ernstig dat het kind in de baarmoeder al overlijdt en de zwangerschap voortijdig tot een einde komt.

DNA-afwijkingen bij de man

De bouwstof van chromosomen is DNA. Soms ontbreekt een stukje DNA van het Y-chromosoom. Men spreekt dan van *Y-deletie*. Deze DNA-afwijking komt voor bij enkele procenten van mannen met verminderde vruchtbaarheid. Een man met een Y-deletie geeft deze afwijking door aan zijn zoon, die dan vrijwel zeker later ook vruchtbaarheidsproblemen zal hebben, al is de ernst daarvan moeilijk te voorspellen.
Een andere DNA-afwijking die voorkomt bij mannen met vruchtbaarheidsproblemen kan wijzen op een verhoogde kans op taaislijmziekte (cystische fibrose). Het is mogelijk dat de man een bepaalde vorm van deze ziekte heeft of het gen voor deze ziekte bij zich draagt (dragerschap). *Een bepaalde vorm van taaislijmziekte* uit zich in een aangeboren afwijking van de zaadleiders. Er zijn dan helemaal geen zaadcellen in het zaad aanwezig. De afwijking van de zaadleiders is hier het enige verschijnsel van de ziekte. Verderop bespreken wij methoden om in zo'n situatie uit de bijbal of de testikel zaadcellen te winnen waarmee men vervolgens ICSI kan uitvoeren. *Dragerschap voor taaislijmziekte* heeft geen betekenis voor iemands gezondheid of vruchtbaarheid. Hoewel men vroeger dacht

dat mannen met vruchtbaarheidsproblemen vaker drager waren, heeft onderzoek uitgewezen dat dragerschap bij hen niet vaker voorkomt dan bij normaal vruchtbare mannen. Daarom doet men in Nederland bij mannen met vruchtbaarheidsproblemen geen routineonderzoek naar dragerschap voor taaislijmziekte.

Onbekende erfelijke factoren

Uit stamboomonderzoek blijkt dat in sommige families vaker mannelijke vruchtbaarheidsproblemen voorkomen dan in andere. Onbekende erfelijke factoren spelen hierbij waarschijnlijk een rol. Toch is niet te voorspellen hoe groot de kans op verminderde vruchtbaarheid is voor een man of jongen uit zo'n familie.

Onderzoek van de man

Alvorens ICSI te starten, adviseren artsen chromosoomonderzoek en soms onderzoek naar een Y-deletie. Dit zijn bloedonderzoeken. De uitslag duurt 6-12 weken. Bij een afwijkende uitslag bespreekt als regel een klinisch geneticus (medisch specialist op het gebied van erfelijke ziekten) de gevolgen, zoals de kans op een aangeboren afwijking of vruchtbaarheidsproblemen voor een zoon. Als de uitslagen normaal zijn, is er geen verhoogde kans op *ernstige* aangeboren afwijkingen of ziekten bij het kind.

MESA en TESE

Hierboven noemden wij reeds de mogelijkheid om zaadcellen uit de bijbal te verkrijgen. Dit gebeurt via een techniek die 'microchirurgische epididymale sperma-aspiratie' (MESA) wordt genoemd. Omdat de risico's van deze behandeling nog niet voldoende bekend zijn, is de MESA-techniek op het tijdstip dat deze folder wordt geschreven (2001) slechts in enkele ziekenhuizen in Nederland toegestaan.

Afwezigheid van de zaadleiders als gevolg van een bepaalde vorm van taaislijmziekte kan een reden zijn voor MESA. Wanneer nu de vrouw draagster is van deze ziekte, dan is de kans groot dat het kind een ernstige vorm van taaislijmziekte krijgt.

Dit geldt ook voor een andere behandeling, testiculaire sperma-extractie (TESE), waarbij men zaadcellen uit de testikel haalt. TESE is in Nederland niet toegestaan. Bij paren die een MESA of TESE-behandeling (in het buitenland) overwegen, doet men vaak voorafgaand in Nederland DNA-onderzoek om te bepalen of de man zo'n vorm van taaislijmziekte heeft en of zijn partner draagster is. Zo is de kans op een kind met taaislijmziekte te voorspellen.

5.5 Alle risico's voor kinderen geboren na ICSI op een rij

Vatten we genoemde risico's samen, en kijken we ook naar ander onderzoek, dan zijn de volgende conclusies mogelijk:

- De kans op een zwangerschap die eindigt met de geboorte van een kind is bij ICSI even groot als bij 'gewone' IVF, ongeveer 15-20% per behandeling.
- Van de zwangerschappen die door ICSI ontstaan eindigt ongeveer 25% in een miskraam. Dit percentage is even hoog als bij IVF.
- De belangrijkste tot nu toe bekende gezondheidsproblemen voor kinderen geboren na ICSI- (en IVF-) zwangerschappen zijn het gevolg van meerlingzwangerschappen. Deze zwangerschappen eindigen vaker in een vroeggeboorte, met meer kans op blijvende handicaps.
- Op dit moment tonen de meeste onderzoeken aan dat ICSI-kinderen niet meer kans op een aangeboren afwijking hebben dan op andere wijze verwekte kinderen. Zo is voor kinderen uit de algemene bevolking de kans op een aangeboren afwijking zo'n 3%. Sommige onderzoekers vinden meer kleine aangeboren afwijkingen na ICSI, maar anderen kunnen dit niet bevestigen.
- Alleen bij een vader met een gebalanceerde translocatie (chromosoomafwijking waarbij twee stukjes van twee verschillende chromosomen van plaats verwisseld zijn), is er een vergrote kans op ernstige aangeboren afwijkingen.
- Bij vaders met een Y-deletie krijgt een zoon vrijwel zeker later ook vruchtbaarheidsproblemen. Maar ook binnen families waar veel mannelijke vruchtbaarheidsproblemen voorkomen, is de kans groot dat zonen eveneens deze problemen zullen hebben, al is geen Y-deletie aangetoond.
- ICSI-kinderen hebben een geringe kans op een chromosoomafwijking waarbij er een extra chromosoom is. Het gaat hierbij niet om een extra chromosoom 21, zoals bij het Downsyndroom, maar om een extra geslachtschromosoom. Deze kans is klein (ongeveer 1%). Kinderen met een extra geslachtschromosoom ontwikkelen zich vrijwel altijd normaal, terwijl ook hun uiterlijk normaal is.
- Ten aanzien van de verstandelijke ontwikkeling beschrijft één onderzoek vooral bij 'ICSI-jongens' een geringe achterstand. Andere onderzoeken bevestigen deze bevindingen niet.
- Net als bij IVF zijn er bij elke ICSI-behandeling onbekende gezondheidsrisico's, die samenhangen met laboratoriumprocedures buiten het lichaam en hormoongebruik rond de bevruchting.

- De gevolgen van het niet plaatsvinden van de 'natuurlijke selectie' bij ICSI zijn onbekend.

Deze gegevens laten zien dat vroeggeboorte bij meerlingzwangerschappen de belangrijkste tot nu toe bekende oorzaak is van mogelijke gezondheidsproblemen van ICSI-kinderen. Naarmate een bevalling vroeger plaatsvindt, is de couveuseopname langduriger en de kans op handicaps of sterfte groter. Evenals niet-ICSI-kinderen hebben ICSI-kinderen ongeveer 3% kans op een aangeboren afwijking.

6 IS ICSI EEN REDEN VOOR EXTRA ONDERZOEK TIJDENS DE ZWANGERSCHAP?

Vrouwen van 36 jaar en ouder kunnen op grond van hun leeftijd overwegen chromosoomonderzoek door middel van een vruchtwaterpunctie of vlokkentest te laten verrichten, omdat zij iets meer kans hebben op een kind met het Downsyndroom. Een kansberekeningtest door middel van bloedonderzoek (tripel-test) of echo (nekplooimeting) behoort eveneens tot de mogelijkheden.

Meer informatie daarover vindt u in de brochure *Prenatale diagnostiek als u 36 jaar of ouder bent*.

Bij ICSI op jongere leeftijd bespreken sommige artsen ook een vlokkentest of een vruchtwaterpunctie, hoewel een vlokkentest moeilijk kan zijn bij een meerling. Beide onderzoeken brengen een kleine kans (0,3-0,5%) op een miskraam mee. Het is van belang te weten dat door ICSI de kans op een kind met een chromosoomafwijking als het Downsyndroom niet verhoogd is.

De licht verhoogde kans (1%) op een kind met een afwijking van de geslachtschromosomen is voor artsen geen reden om een abortus te bespreken of te adviseren. Er zijn dus eigenlijk geen goede redenen om alleen in verband met ICSI een vlokkentest of vruchtwaterpunctie te doen. Deze onderzoeken geven geen antwoord op alle genoemde onzekerheden die met een ICSI-behandeling gepaard gaan. Ouders die kiezen voor ICSI, kiezen daarmee welbewust voor een aantal onzekerheden over langetermijngevolgen voor hun kind en voor de vrouw. Zeker bij paren die sterk naar een kind verlangen is dit een moeilijke keuze, die u gerust met de arts kunt bespreken.

Toch kan welbewust kiezen voor 'het onzekere' eraan bijdragen dat u zich later niet afvraagt: 'Waarom is mij dit niet verteld?'

7 VERSCHILLENDE ONDERZOEKEN TIJDENS DE ZWANGERSCHAP OP EEN RIJ

De hieronder genoemde onderzoeken zijn tijdens de zwangerschap mogelijk en worden vaak vergoed. Veel artsen twijfelen aan het nut van deze onderzoeken, als ze alleen worden gedaan omdat er een ICSI-behandeling heeft plaatsgevonden.

7.1 Uitgebreid echoscopisch onderzoek

Bij dit onderzoek beoordeelt men de verschillende organen van het kind bij 18-20 weken zwangerschap. Ernstige, grote aangeboren afwijkingen ziet men hierbij doorgaans wel, maar ICSI-kinderen hebben niet meer kans op dit soort afwijkingen. Kleinere, meestal minder ernstige aangeboren afwijkingen ziet men bij dit onderzoek snel over het hoofd.

7.2 Vlokkentest of vruchtwaterpunctie

Bij deze onderzoeken vindt chromosoomonderzoek van het kind plaats. Het is de vraag of deze onderzoeken bij ICSI zinvol zijn. De kans op een chromosoomafwijking als gevolg van ICSI is gering (1%). Bovendien betreft het hier meestal een geslachtschromosoomafwijking, die geen ernstige verschijnselen geeft. Ook moet u rekening houden met een kans op een miskraam van een in principe goede zwangerschap als gevolg van een vlokkentest (0,5%) of een vruchtwaterpunctie (0,3%). Meer informatie over de vlokkentest en de vruchtwaterpunctie vindt u in de brochure *Prenatale diagnostiek bij aangeboren of erfelijke aandoeningen*. In die brochure staat ICSI overigens niet vermeld als reden voor zo'n onderzoek. De informatie over de tripel-test en de nekplooimeting gaat over de kans op een kind met het Downsyndroom; deze kans is niet verhoogd bij ICSI. De tripel-test en de nekplooimeting zeggen weinig over de kans op geslachtschromosoomafwijkingen.
Bij ouders die zelf een chromosoomafwijking hebben is de situatie anders; voor hen is het vaak wel zinvol een vlokkentest of een vruchtwaterpunctie te overwegen.

8 ONDERZOEK BIJ KINDEREN DIE NA ICSI GEBOREN ZIJN

Op dit ogenblik vindt in Nederland geen onderzoek op latere leeftijd plaats van alle ICSI-kinderen. Misschien gebeurt dit wel in de toekomst. De mogelijkheid bestaat dat het ziekenhuis waar de ICSI plaatsvond, u te zijner tijd benadert om mee te doen aan onderzoek van uw kind(eren). Waarschijnlijk gaat het dan om eenvoudig onderzoek naar de gezondheidstoestand, de verstandelijke ontwikkeling en het gedrag, waaronder de

manier van bewegen. Medewerking is nooit verplicht, maar wordt wel op prijs gesteld om een beter antwoord op bovengenoemde onzekerheden te geven.

9 MEER INFORMATIE OVER ICSI

Meer informatie is te verkrijgen bij Freya, Patiëntenvereniging voor vruchtbaarheidsproblematiek, Postbus 476, 6600 AL Wijchen; tel. (024) 645 10 88, fax (024) 645 46 05; website www.freya.nl ; e-mail secretariaat@freya.nl . Als zwanger worden niet vanzelf gaat. Cd-rom, verkrijgbaar in de boekhandel. NVOG-VSOP-brochures: *Prenatale diagnostiek als u 36 jaar of ouder bent Prenatale diagnostiek bij aangeboren of erfelijke aandoeningen.*

Summary

Introduction

In the Netherlands, every one out of seven couples does not achieve a pregnancy within one year of active sexual intercourse. This subfertility may have various causes in either partner. According to the World Health Organization (WHO), in 20 percent of all cases the problem is predominantly male and in 38 percent predominantly female. Male subfertility is frequently associated with a gross reduction in the number of sperm (oligozoospermia) and/or the abnormal motility of sperm (asthenozoospermia) and/or with abnormal morphology of the sperm (teratozoospermia). Mostly, all these parameters are impaired in male subfertility, so called **OAT** = **O**ligo**A**sthenо**T**eratozoospermia.

The cause of this male subfertility remains unknown in 40% of all cases. Recently, much attention has been given to genetic causes of male subfertility such as chromosomal aberrations, microdeletions of the Y chromosome and other possible monogenetic causes of male subfertility. This attention is stimulated by the increasing knowledge of genetics and the recently finished documentation of the human genome, called the Human Genome Project. On the other hand people are concerned that via intracytoplasmic sperm injection (ICSI) there is a potential transmission of genetic defects to the offspring.

In this thesis we have tried to answer the following questions:

The first part - **Part I** - of this thesis describes the familial occurrence of male subfertility. We continue in **Part II** with the molecular genetic search for genes involved in male subfertility. In this strategy we have used both linkage analysis as candidate gene research. In the last and third part of this thesis - **Part III** - we present three studies on the clinical implications of the genetic aspects involved in male subfertility.

Part I

In **chapter 1** the survey on the familial occurrence of male subfertility is presented. Surprisingly the frequency of subfertility among relatives was reported lower than among the responders that filled out the questionnaire and their family tree. Probably the frequency of fertility problems is underestimated among relatives. Knowledge on fertility problems travels selectively among families causing substantial misclassification. Male subfertility is a difficult subject to discuss and gives rise to this **"Taboo Bias"**.

The next **chapter, 2,** describes the familial occurrence of male subfertility among relatives of subfertile men. The frequency of male subfertility is increased among brothers and maternal uncles of subfertile men. Besides the hypothesized autosomal inheritance in male subfertility, the higher frequency of male subfertility among maternal uncles may be due to X-linked inheritance.

These subfertile men with a positive family history more often have normal serum levels of FSH and LH. This can indicate a disturbed spermatogenesis and an intact hormonal feedback mechanism. Another clinical phenomena is the diminished motility of spermatozoa in this group of men with a possible genetic origin of their fertility problem.

No final conclusions can be drawn, because of the taboo bias described in chapter 1. The differences found between the family history of the subfertile men and their controls may also be caused by recall bias. In other words, subfertile men may be better informed on fertility problems in their families than in our survey.

Part II

In **chapter 3** an X-linkage analysis is described in a family of a subfertile men with 5 subfertile maternal nephews. The pedigree suggested an X-linked inheritance, but this could not be confirmed by molecular linkage analysis. We hypothesised an autosomal dominant trait with sex limited expression.

In **chapter 4 and 5** we present the results on the role of respectively the candidate genes *DAZLA* and the *Androgen receptor gene*. This *DAZLA* gene showed no pathogenic mutations in subfertile men. For the *Androgen receptor gene* we could not confirm the controversial results on the expansion of the CAG repeat length.

Chapter 6 is a completely different kind of molecular genetic research in the discussion on genetic disorders in male subfertility and its clinical implications. Mutations in mitochondrial DNA of subfertile men are described. If these subfertile men father children via ICSI these children might be at risk for a mitochondrial disease. In collaberation with an English research institute this risk seems limited. No transmission of paternal mitochondrial DNA via ICSI was detected.

Part III

In the last part of this thesis we describe some clinical consequences of the genetic aspects of male subfertility.

In **chapter 7** it is shown that 79% of the men with a microdeletion of the Y chromosome choose ICSI. This means that they accept the risk that their son(s) probably develop(s) an impaired spermatogenesis and a fertility problem. This choice is mainly influenced by: (a) the opinion of the counsellor if Y deletions are a serious defect, (b) the fertilisation techniques available in the department and (c) if the counsellor considers his counselling direct.

Chapter 8 shows that subfertile men with a microdeletion of the Y chromosome can influence the treatment. Our results show a decreased fertilisation rate and embryo quality after ICSI using sperm from these men. This may also have an effect on the pregnancy rate and take home baby rate, but larger numbers of couples are required to study the possible prognosis of such deletions on ICSI treatment. The last **chapter, 9,** of this thesis confirms earlier and later results on the follow-up of ICSI. We have observed no statistical increase of abnormalities after ICSI for: preembryonic development, obstetric and neonatal outcome and follow-up of children born after ICSI. At this, moment ICSI seems a successful and safe technique. However, a prospective follow-up study on ICSI and appropriate control groups will hopefully confirm present results.

Main conclusions

This thesis shows clinical evidence for still unknown genetic factors involved in male subfertility. Up till now, our molecular genetic search for a new gene involved in male subfertility has been unsuccessful. Future research may be successful in the search among this heterogeneous disorder of male subfertility. The next step will be to supply the optimal and objective information for subfertile men and their partners.

Samenvatting

Introductie

Een op de zeven paren in Nederland kan binnen een jaar van onbeschermde samenleving geen zwangerschap bewerkstelligen. Deze zogenaamde subfertiliteit kan verschillende oorzaken hebben bij zowel man als vrouw. Volgens de wereld gezondheidsorganisatie (WHO) bestaat er in 20% van de gevallen een mannelijke oorzaak en in 38% van de gevallen is er met name een vrouwelijke oorzaak voor de subfertiliteit. De mannelijke subfertiliteit wordt meestal veroorzaakt door een verminderde zaadkwaliteit. Zo kunnen er zich te weinig zaadcellen in de zaadlozing bevinden (oligozoöspermie), een verminderde beweeglijkheid van zaadcellen optreden (asthenozoöspermie) en/of een te hoog percentage van afwijkende vormen van zaadcellen voorkomen (teratozoöspermie). Vaak zijn alle bovengenoemde parameters verminderd en dit wordt dan afgekort tot **OAT** = **O**ligo**A**sthenо**T**eratozoöspermie.
De oorzaak van deze verminderde zaadkwaliteit blijft echter vaak onbekend. De laatste jaren bestaat er veel aandacht voor de genetische oorzaken van deze mannelijke subfertiliteit zoals (1) chromosomale afwijkingen, (2) microdeleties van het Y chromosoom en (3) andere mogelijke monogenetische oorzaken van mannelijke subfertiliteit. Deze aandacht voor genetische oorzaken wordt enerzijds gestimuleerd door de toename in kennis van de genetica en de recente afronding van het in kaart brengen van het menselijke genetische materiaal, het Human Genome Project. Anderzijds is er bezorgdheid rondom de overdracht van genetische afwijkingen aan het nageslacht door de nieuwe voortplantingstechniek van intracytoplasmatische sperma injectie (ICSI).
Daarom hebben wij getracht in dit proefschrift een aantal vraagstellingen te beantwoorden:
In het eerste deel **- Deel I -** van dit proefschrift wordt het onderzoek beschreven naar het familiair voorkomen van mannelijke subfertiliteit. Vervolgens worden er in het tweede deel **- Deel II -** een aantal studies gepresenteerd van onze moleculair genetische zoektocht naar mogelijke genen die betrokken zijn bij mannelijke subfertiliteit. Hierbij hebben we zowel koppelingsonderzoek als mutatieonderzoek in kandidaatgenen toegepast.
In het laatste en derde deel - **Deel III -** van dit proefschrift onderzochten wij een aantal klinische implicaties van de gevonden genetische afwijkingen.

Deel I

In **hoofdstuk 1** zijn de resultaten beschreven van het populatieonderzoek naar het familiair voorkomen van mannelijke subfertiliteit. Opvallend resultaat van deze studie is dat de frequentie van vruchtbaarheidsproblemen bij familieleden lager is dan bij degene die de vragenlijst invulde. De frequentie van vruchtbaarheidsproblemen bij familieleden wordt dus onderschat. Dit kan hoogstwaarschijnlijk worden verklaard doordat men in families niet volledig op de hoogte is van elkaars vruchtbaarheidsproblemen. Mannelijke vruchtbaarheidsproblemen is een moeilijk bespreekbaar onderwerp en geeft dus mogelijke aanleiding voor deze **"Taboe Bias"**.

Het volgende **hoofdstuk, 2,** beschrijft het familiair voorkomen van mannelijke vruchtbaarheidsproblemen. Broers en ooms aan moederszijde van subfertiele mannen lijken vaker vruchtbaarheidsproblemen te ondervinden. Naast de eerder gesuggereerde autosomale overerving van mannelijke subfertiliteit kan de hogere frequentie van vruchtbaarheidsproblemen van ooms aan moederszijde en broers ook wijzen op geslachtsgebonden overerving. Bovendien zijn de serumwaarden van het Follikel Stimulerend Hormoon en het Luteinizerend hormoon bij mannen met zo'n positieve familieanamnese statisch lager en vallen vaker binnen de normaalwaarden. Dit kan wijzen op een intact endocrienologisch feedback mechanisme bij een gestoorde spermatogenese. Daarnaast is de motiliteit van de zaadcellen lager, in deze groep mannen met een mogelijke genetische oorsprong voor hun vruchtbaarheidsprobleem.

Definitieve conclusies kunnen echter nog niet worden getrokken. In hoofdstuk 1 beschreven we al de taboe bias. Het verschil in familiair voorkomen van mannelijke subfertiliteit kan mogelijk ook worden verklaard door het beter op de hoogte zijn van dergelijke problemen in families van de subfertiele man, zogenaamde recall bias.

De klinische karakteristieken van subfertiele mannen met een positieve familieanamnese vertonen vaker bovengenoemde klinisch statistische verschillen, maar de groep is fenotypisch heterogeen.

Deel II

In **hoofdstuk 3**, wordt het koppelingsonderzoek van het X chromosoom beschreven van de familie van een subfertiele man met 5 subfertiele neven. De stamboom suggereert

duidelijk een geslachtsgebonden overerving bij deze subfertiele mannen die allen een zeer slechte zaadkwaliteit hebben. Dit kon met moleculair genetisch onderzoek niet worden bevestigd en daarom hebben we geconcludeerd dat er mogelijk sprake is van autosomale dominante overerving met geslachtsgebonden expressie.
In de **hoofdstukken 4 en 5** presenteren we respectievelijk de resultaten van de mogelijke rol van het *DAZLA* gen en het *Androgeen receptor* gen bij mannelijke subfertiliteit. In het DAZLA gen konden geen pathogenetische mutaties worden aangetoond. In het onderzoek van de androgen receptor konden wij de controversiële resultaten betreffende de toename van de lengte van CAG repeats ook niet bevestigen.
Hoofdstuk 6 vormt een geheel ander moleculair genetisch onderwerp bij de discussie rondom genetisch afwijkingen bij mannelijke subfertiliteit en de mogelijke klinische consequenties. Er zijn namelijk mutaties beschreven in het mitochondriaal DNA van subfertiele mannen. Dit kan een risico vormen, omdat deze mannen tegenwoordig door ICSI kinderen kunnen voortbrengen. In deze gezamenlijke studie met een Engelse onderzoeksgroep zijn er echter geen aanwijzingen gevonden voor paternale transmissie van mitochondriaal DNA door ICSI.

Deel III

In het laatste deel van dit proefschrift beschrijven we een aantal klinische consequenties van de genetische aspecten van mannelijke vruchtbaarheidsproblemen. In **hoofdstuk 7** wordt getoond dat 79% van de mannen met een microdeletie van het Y chromosoom toch kiezen voor een ICSI behandeling. Zij aanvaarden hierbij dus het risico dat zonen later misschien ook vruchtbaarheidsproblemen krijgen. Deze keuze werd met name beïnvloed door: (a) de mening van de voorlichter over de ernst van microdeleties op het Y chromosoom, (b) het alleen maar voor handen zijn van donor inseminatie op de afdeling van de voorlichter en (c) de directheid van het gesprek door de voorlichter.
Hoofdstuk 8 laat zien dat het hebben van zo'n microdeletie op het Y-chromosoom ook zijn invloed kan uitoefenen op de behandeling. Zo bleek dat bij deze zeldzame aandoening de fertilisatiegraad en embryokwaliteit verlaagd zijn. Mogelijk heeft dit ook effect op de zwangerschapskans, maar door de kleine onderzoeksgroep kan hier nog geen uitspraak over gedaan worden.

Het laatste **hoofdstuk, 9,** van dit proefschrift bevestigt de resultaten van de follow-up gegevens van eerder en later verschenen onderzoek. ICSI lijkt op dit moment een succesvolle en veilige techniek. Echter prospectief onderzoek met een zo adequaat mogelijke controlegroep laat hier hopelijk ook op langere termijn geruststellende resultaten van zien.

Conclusies

In dit proefschrift zijn er dus aanwijzingen gevonden voor de mogelijke genetische origine van mannelijke subfertiliteit. De moleculair genetische zoektocht naar een nieuw gen dat hierbij betrokken is heeft echter nog niet tot een nieuwe doorbraak geleid.
Toekomstig onderzoek zal hopelijk leiden tot een aantal belangrijke genen die betrokken zijn bij de heterogene aandoening van mannelijke subfertiliteit. Het is dan voorts belangrijk dat al deze informatie beschikbaar moet zijn voor de mannen met een vruchtbaarheidsprobleem.

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Curriculum Vitae

Ron van Golde, born in Maastricht (1971), graduated from the 'Stedelijk Scholengemeenschap' in Maastricht before attending medical school at the University of Maastricht (1990-1997). During his time in medical school, he worked as a researcher in Barcelona during two different time periods. In 1994, he worked at the Universitat Autonoma de Barcelona, studying the effect of acid Tyrode's as used in Preimplantation Genetic diagnosis. Before graduating from medical school he worked at the Institut Universitaria Dexeus, Barcelona performing a follow-up study on intracytoplasmic sperm injection.

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